On the diversification of highly host-specific symbionts: the case of feather mites

Jorge Doña

PhD Thesis

On the diversification of highly host-specific symbionts: the case of feather mites

Memoria presentada por el Licenciado en Biología y Máster en Genética y Evolución Jorge Doña Reguera para optar al título de Doctor por la Universidad de Sevilla

Fdo. Jorge Doña Reguera

Conformidad de los directores:

Director
Fdo.: Dr. Roger Jovani Tarrida

Director
Fdo.: Dr. David Serrano Larraz

Tutor
Fdo.: Dr. Manuel Enrique Figueroa Clemente

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- **Chapter 4**: Doña, J., Potti, J., De la Hera, I., Blanco, G., Frias, O., and Jovani, R. (2017). Vertical transmission in feather mites: insights into its adaptive value. *Ecological Entomology*, **42**, 492-499.
- **Chapter 5**: Doña, J., Proctor, H., Serrano, D., Johnson, KP., Oddy-van Oploo, A., Ascunce, MS., Huguet-Tapia, JC., Jovani, R. (2018). Feather mites play a role in cleaning host feathers: New insights from DNA metabarcoding and microscopy. *Molecular Ecology*, *in press*.
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- **Chapter 8**: Doña, J., Serrano, D., Mironov, S., Montesinos-Navarro, A, Jovani, R. Chasing host-switches: A DNA metabarcoding study on the eco-evolutionary scenario of host-shift speciation of host-specific symbionts (under review in *Molecular Ecology* —invited article).
- **Appendix 1**: Vierna, J.*, Doña J.*, Vizcaíno, A., Serrano D and Jovani, R. (2017). PCR cycles above routine numbers do not compromise high-throughput DNA barcoding results. *Genome*, **60**, 868-873.
- **Appendix 2**: Mironov, S., Doña, J., Jovani, R. (2015). A new feather mite of the genus *Dolichodectes* (Astigmata: Proctophyllodidae) from *Hippolais polyglotta* (Passeriformes: Acrocephalidae) in Spain. *Folia Parasitologica*, **62**, 32.

- **Appendix 3**: Esteban, R., Doña, J., Vierna, J., Vizcaíno, A., Serrano, D., Jovani, R. (2018). The complete mitochondrial genome of the feather mite *Trouessartia rubecula* Jablonska, 1968 (Astigmata: Analgoidea: Trouessartiidae). *Mitochondrial DNA Part B: Resources*, **3**, 652-654.
- **Appendix 4**: Jovani, R., Doña J., Labrador, M and Serrano D. (2017). Opening the doors of parasitology journals to other symbionts. *Trends in Parasitology*, **33**, 578-579.

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- Doña, J.*, Moreno-García M.*, Criscione CD., Serrano, D., Jovani, R. (2015). Species mtDNA genetic diversity explained by infrapopulation size in a host-symbiont system. *Ecology and Evolution*, **5**, 5801-5809.
- Carlson, C., Burgio, K., Dougherty, E., Phillips, A., Bueno, V., Clements, C., Castaldo, C., Dallas, T., Cizauskas, C., Cumming, C., Doña, J., Harris, N., Jovani, R., Mironov, S., Muellerklein, O., Proctor, H., Getz, W. Parasite biodiversity faces extinction and redistribution in a changing climate. *Science advances*, **3**, e1602422.
- De Rojas, M., Doña, J., Jovani, R., Dimov, I., Zurita, A., Callejón, R., Rodríguez-Plá, M., Evidence of cryptic species in the genus *Tinaminyssus* (Acari: Rhinonyssidae) based on morphometrical and molecular data. *Experimental and Applied Acarology*, *in press*.

* Authors contributed equally to this work

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Abstract

One of the most relevant and poorly understood topics in Evolutionary Ecology is symbiont evolutionary diversification. Since Fahrenholz's rule (1913), the idea of symbionts speciating following hosts speciation (i.e., cospeciating) has been pervasive. Recent studies, however, have shown that host-shift speciation (speciation after switching to a new host) is almost as relevant as cospeciation in explaining symbiont diversification. Also, these studies have revealed that methodological biases have favored cospeciation. Nonetheless, most symbiont groups, especially those highly host-specific and specialized in which cospeciation is expected to be the rule, such as the feather mites of birds, were yet to be studied.

Symbionts are the most abundant and diverse organisms on Earth, and thus essential components of ecosystems. However, symbionts have attracted historically less attention than other organisms and their study entails numerous methodological challenges, so surprisingly little is understood about the basic biology and ecology of many symbiont groups, especially the non-parasitic. By studying vane-dwelling feather mites living permanently on the surface of flight feathers of birds (Acariformes: Astigmata: Analgoidea and Pterolichoidea), this thesis is a contribution to fill this gap.

This thesis is divided into three parts: 1) First, resources and molecular tools enabling large-scale studies of feather mites are developed. 2) Then, these and other tools are used to investigate eco-evolutionary aspects relevant to understand feather mite diversification, such as their mode of transmission and the type of interaction they have with their hosts. 3) Finally, feather mites diversification at a macro- and microevolutionary scale is investigated.

The first part compiles a global database of bird-feather mites associations. Also, it evaluates and adjusts DNA barcoding and metabarcoding to be suitable methodologies for studying feather mites. The second part reveals feather mites as highly specialist and host-specific symbionts whose main mode of transmission is vertical. Analyses of feather mites diet reveal them as trophic generalists which maintain a commensalistic-mutualistic

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relationship with birds. Finally, the last part of the thesis shows host-shift speciation as the primary process driving the diversification of feather mites. Also, it highlights that major-host switching, despite being an infrequent process, is highly relevant for the diversification of this group. Lastly, analyses of straggling reveal a high rate of preferential straggling governed by ecological filters.

Overall, despite feather mites are revealed as highly specialized and host-specific symbionts, the coevolutionary scenario is highly dynamic. Straggling and host-switching are prevalent processes which allow colonizing new hosts in highly specialized and host-specific symbionts. Accordingly, coevolution and codiversification do not operate in isolated host-symbiont interactions but more likely in a manner compatible with the geographic mosaic of coevolution. Finally, ecological fitting and interspecific competition are most likely the main factors governing the (co)eco-evolutionary dynamics.

Resumen

La diversificación evolutiva de los simbiontes es uno de los aspectos más relevantes, pero menos entendidos en Ecología Evolutiva. Desde la regla de Fahrenholz (1913), la idea de que los simbiontes especian a la par que sus hospedadores (i.e. coespecian) ha sido extremadamente popular. Sin embargo, estudios recientes han encontrado que la especiación por salto de hospedador (el proceso de especiación que ocurre cuando los simbiontes especian a consecuencia de un cambio de hospedador) es casi tan relevante como la coespeciación. Estos estudios, además, han encontrado que problemas metodológicos favorecían que se encontraran evidencias de coespeciación donde no las había. En cualquier caso, los procesos de diversificación evolutiva de la mayoría de los grupos de simbiontes nunca han sido investigados. Especialmente de aquellos altamente especializados y específicos en términos de hospedador, que son aquellos donde el proceso de coespeciación se espera que sea más relevante, como los ácaros de las plumas de las aves.

Los organismos simbiontes son el grupo más abundante y diverso de la tierra y, por ende, son componentes esenciales de los ecosistemas. Sin embargo, históricamente los simbiontes han atraído menos la atención de los investigadores, en parte debido a que su estudio conlleva numerosos retos metódologicos. De hecho, debido a esto, actualmente se desconoce una gran parte de aspectos sobre su biología básica y ecología, especialmente de aquellos simbiontes no parásitos. Ésta tésis pretende completar este vacío de conocimiento mediante el estudio de los ácaros de las plumas de las aves.

La tésis está dividida en tres partes: 1) En la primera parte se han generado recursos y herramientas moleculares para estudios a gran escala en este grupo de simbiontes. 2) Despues, éstas y otras herramientas se han usado para investigar aspectos eco-evolutivos relavantes para entender el proceso de diversificación evolutiva, tales como, el modo de

transmisión y el tipo de interacción que mantienen con sus hospedadores. 3) Finalmente, se ha estudiado el proceso de diversificación evolutiva a escala macro y microevolutiva.

La primera parte de la tesis presenta una base de datos global de relaciones ácaro-ave resultado de una extensa compilación de datos ya presentes en la literatura. También evalua y ajusta metodologías de "DNA barcoding" y "metabarcoding" para el estudio de los ácaros de las plumas. La segunda parte, revela a los ácaros de las plumas como simbiontes altamente especialistas en términos de hospedador cuyo modo de transmisión principal es el vertical. Por otro lado, el análisis de la dieta de los ácaros los sitúa como simbiontes comensales-mutualistas de las aves. Finalmente, la ultima parte de la tesis demuestra que la especiación por salto de hospedador es el proceso principal de diversificación de este grupo de simbiontes. Asimismo, también demuestra que los saltos de hospedador a larga distancia, a pesar de ser muy raros son muy relevantes para la diversificación de este grupo. Por último, los análisis de simbiontes encontrados en hospedadores inesperados ("stragglers") revelan que este proceso es más prevalente de lo que se pensaba, y que sigue un patrón compatible con que está modulado por filtros ecológicos.

A pesar de que los ácaros de las plumas se revelan como altamente especializados y específicos en términos de hospedador, su escenario coevolutivo es muy dinámico. El proceso de "straggling" y de cambio de hospedador son procesos prevalentes que permiten colonizar nuevos hospedadores. De acuerdo con esto, los procesos de coevolución y codiversificación en estos organismos no operan de manera aislada para cada pareja de hospedador y simbionte, si no de una manera similar a un mosaico geográfico de coevolución. Finalmente, el encaje ecológico y la competencia intraspecífica se identifican como los factores potencialmente más relevantes en las dinámicas (co)eco-evolutivas.

Introduction

The history of biological diversity is strongly influenced by the fact that species do not live in isolation (Thompson, 1994, 2005, 2009; Clayton et al. 2016). Darwin (1859), already envisioned ecological interactions as drivers of biodiversity in his famous paragraph about the entangled bank. Nowadays, this conception has been largely supported, to the point that the history of life now makes no sense without considering the interactions between species (Price, 2002; Thompson, 2009, 2014; Clayton et al. 2016; Plotkin, 2017). This is especially true in host-symbiont systems.

Symbionts (i.e., parasites, mutualists and commensalists that intimately interact with their hosts; Leung & Poulin, 2008) are the most abundant and diverse organisms on Earth (Price 1980; Dobson et al. 2008; Morand, 2015; Larsen et al. 2017). Symbionts are present in almost every ecosystem, in which they mediate in several bottom-up and top-down processes, and thus are essential for ecosystem functioning and stability (Hudson et al. 2006; Hatcher et al. 2012; Bronstein, 2015; Werner, 2018). For instance, parasites alone can constitute more than 75% of the total links of food webs (Lafferty et al. 2006, 2008). Symbionts are also involved in areas with a high economic impact, such as plagues, emerging infectious diseases (Hoberg & Brooks, 2015), biological control (Lacey, 2001), biological invasions (Dunn, 2015), and biotic responses to climate change (Carlson et al. 2017).

Symbionts diversification:

The degree of intimacy and dependence that most host-symbiont interactions present could lead them to coevolve (Thompson, 2005; Clayton et al. 2016). Hence,

according to the numbers of symbionts species, coevolution is now considered as one of the most relevant processes shaping the history of life (Thompson, 2009; 2014). Understanding symbiont diversification needs understanding host-symbiont coevolution. Coevolution, when not defined in the strict sense (i.e., coadaptation, which requires of reciprocal adaptation between interacting species, Janz, 2011; Althoff et al. 2014), is the evolution of one species in response to its interaction with another species (Futuyma, 2013; Clayton et al. 2016). Coevolutionary interactions are spatially structured, as stated by the geographic mosaic theory of coevolution (Thompson, 2005). Moreover, these coevolutionary interactions then translate into broad patterns of diversification, strongly influenced by dispersal (or transmission when referring to parasites) and selection processes (Thompson, 2005; Clayton et al. 2016). However, how these coevolutionary interactions scale up to diversification is one of the least understood aspects of evolutionary biology (Clayton et al. 2016).

At the macroevolutionary scale, studies have identified different processes and macroevolutionary events which shape symbiont diversification (Johnson & Clayton, 2004; Clayton et al. 2016). Five main processes and events govern symbiont diversification: cospeciation, host-shift speciation, duplication, sorting, and cohesion (Johnson & Clayton, 2004; Clayton et al. 2016). Among these, cospeciation and host-shift speciation are those with a higher prevalence and relevance on symbiont diversification (de Vienne et al. 2013; Clayton et al. 2016). The first process, cospeciation, is the synchronous speciation of ecologically interacting groups (Clayton et al. 2016). Host-shift speciation, by contrast, can be defined as symbiont speciation after the colonization of a new host (Johnson & Clayton, 2004). Cospeciation and host-shift speciation likely occur in most host-symbiont systems but represents the ends of a continuum in which symbiont eco-evolutionary traits

control the prevalence of one process versus the other (Clayton et al. 2016). For instance, highly specialized and host-specific symbionts in which reproduction is strongly tied to that of its host and vertical transmission often dominates (e.g., the Physconelloides genus of feather lice; Johnson & Clayton, 2004) are prone to cospeciate. On the other hand, symbionts with better transmission capabilities are likely to diversify by host-switching (e.g., primate lentiviruses; Charleston & Robertson, 2002). Outstandingly, a recent review has questioned the real incidence of cospeciation, showing that host-shift speciation indeed explains overall most symbiont diversification and revealing that methodological biases have favored finding cospeciation (de Vienne et al. 2013). Notwithstanding, most symbiont groups, especially those highly host-specific and specialized in which cospeciation is more expected, are yet to be studied. In the same vein, data on dispersal and selection for these groups are usually not available but required to understand coevolutionary dynamics in both micro- and macroevolutionary time (Boulinier et al. 2001). Accordingly, a revisitation of the diversification history of highly host-specific and specialized symbionts which integrates ecological and evolutionary data is needed. This thesis aims to understand at a micro- and macroevolutionary scale the diversification history of a highly specialized and host-specific symbiont group, the feather mites of birds.

Studying symbionts:

Symbiont taxa, especially the non-parasitic, are comparatively poorly understood compared to other organisms (Duarte Rocha et al. 2016; Poulin & Presswell, 2016; Tripp et al. 2017). Part of the explanation comes from the fact that the study of

symbionts entails numerous methodological challenges. Symbionts usually are near-microscopic (or tiny) organisms which usually present morphological characters that are inconspicuous or insufficient for identification (Littlewood, 2011; Perkins et al. 2011; Gómez & Nichols, 2013). Also, they are challenging to keep in the laboratory without their hosts, and problematic for DNA-based methods (Perkins et al. 2011; Gómez & Nichols, 2013). Accordingly, the state of the taxonomy of most symbionts groups is far less advanced than usual for free-living organisms (Littlewood, 2011; Nadler & Pérez-Ponce de León, 2011; Poulin & Presswell, 2016; Troudet et al. 2017). Also, resources such as centralized repositories of symbiont data are hence less abundant than in free-living organisms. Overall, these methodological constraints have primarily hampered the knowledge about symbionts in many areas (Poulin, 2011; Duarte Rocha et al. 2016).

Therefore, in many topics, studies on symbionts require for an extra prior methodological development and the creation of dedicated data repositories before achieving the desired research goal. For instance, the study of symbionts response under a climate change scenario came 13 years after a similar study but on free-living organisms. Further, this study required for a significant collaborative effort between multiple research groups (Carlson et al. 2017).

Recent advances in eDNA methods jointly with the advent of high-throughput sequencing are promising for symbiont studies (Taberlet et al. 2012; Baker et al. 2016). For instance, DNA barcoding and metabarcoding are advancing taxonomy while generating useful ecological data in several free-living groups (Besansky et al. 2003; Hebert et al. 2005; Miller, 2007; Schlick-Steiner et al. 2010; Hajibabaei, 2012; Cristescu, 2014). Consequently, these DNA-based methods are suitable candidates to overcome most of the significant challenges of studying symbionts stated above

(Perkins et al. 2011; Baker et al. 2016). However, these methods are still relatively new and require previous tests and significant methodological advance before being used in specific groups (Smith et al. 2008; Perkins et al. 2011; Coissac et al. 2012). For instance, some symbionts particularities, such as the poor alpha-taxonomy, the lack of DNA barcodes libraries, or the little amount of DNA they usually contain, can have a substantial impact success of these methods (Nadler & Pérez-Ponce de León, 2011; Baker et al. 2016; Allen et al. 2017; Troudet et al. 2017). Therefore, while promising, these methods still need to be adjusted and adequately tested before being used for symbionts studies.

Feather mites as study model:

Birds are inhabited by a rich community of mite symbionts (Walter & Proctor, 2013). Some live on the surface of feathers while others inhabit the skin, nostrils, and respiratory passages (Dabert & Mironov, 1999; Proctor, 2003). This diversity has led some authors to compare this mite community with a jungle in which some mites would live in the branches (feather surface), some on the ground (skin), others in the tree trunks (feather rachis) and finally, others infesting the roots (the feather follicles) (Gaud & Atyeo, 1996; Walter & Proctor, 2013). This thesis is about vane-dwelling feather mites (hereafter, feather mites) (Acariformes: Astigmata: Analgoidea and Pterolichoidea), that is, those that live on the flight feathers of birds (Proctor, 2003).

Feather mites are likely the most species-rich group of ectosymbionts of birds (Proctor & Owens, 2000; Dabert, 2004). They are permanent symbionts, i.e., they develop their entire life-cycle on its bird host (Dabert & Mironov, 1999; Proctor, 2003). Notably, this high degree of dependence makes feather mites good models for studies on coevolutionary and codiversification dynamics (Proctor, 2003; Proctor & Owens, 2000). However, as with many other symbionts, surprisingly little is understood about their basic biology and ecology (Proctor, 2003; Proctor & Owens, 2000). One of the main reasons for this poor understanding

is that they are problematic for large-scale ecological studies as species-level identifications are laborious even for specialized taxonomists (Proctor, 2003). Thus, some fundamental aspects of their ecology and evolution which are essential to understand their coevolutionary dynamics and the drivers of their diversification are still unclear.

As stated above, transmission and selection are the two most important processes influencing the geographic mosaic of coevolution and thus essential to understand symbiont diversification (Thompson, 2005; Clayton et al. 2016). In feather mites, vertical transmission is presumed to be the main mode of transmission albeit it has not been yet experimentally demonstrated. This is important as vertically transmitted symbionts are expected to coevolve and strictly cospeciate with their hosts (Clayton et al. 2016). Similarly, whether they are parasites, mutualists or commensalists is a necessary ingredient to understand how natural selection is operating in this system. That is, if they are parasitic or mutualistic, reciprocal selection and thus coadaptive diversification (i.e., diversification of one lineage in response to reciprocal selection between interacting lineages; Althoff et al. 2014) may have taken place. However, if they are commensalistic, only unidirectional selection is expected, and thus adaptive codiversification (i.e., correlated diversification of interacting lineages in response to unidirectional selection on one of the lineages; Clayton et al. 2016) is the diversification process expected. Previous studies suggest that feather mites coevolve and cospeciate with their hosts (Dabert & Mironov, 1999; Dabert, 2004). However, only a few studies on their diversification are available, and these studies were restricted in scope and limited by the lack of eco-evolutionary knowledge (Proctor & Owen, 2004). Therefore, a general understanding of the coevolutionary dynamics and diversification history of the group is lacking.

Objectives

In the first part of the thesis, resources and molecular tools for studying feather mites are developed.

- **Chapter 1** compiles an extensive and centralized dataset of associations between feather mites and birds.
- **Chapter 2** tests DNA barcoding usefulness as a molecular tool to identify feather mites and for the integrative taxonomy of these symbionts.
- **Chapter 3** develops a DNA metabarcoding pipeline to accurately identify and quantify the feather mite species present in a complex sample.
  - o **Appendix 1** investigates whether increasing the number of PCR cycles impacts negatively on the outcome of high-throughput DNA barcoding.
  - o **Appendix 2** describes a new feather mite species from the genus *Dolichodectes*
  - o **Appendix 3** presents the complete mitochondrial genome of a feather mite species.

In the second part of the thesis, eco-evolutionary aspects of feather mites relevant to understand their diversification are investigated.
- **Chapter 4** investigates the transmission dynamics of feather mites.
- **Chapter 5** investigates the nature of the relationship between birds and feather mites.
  - o **Appendix 4** exposes the benefits of studying symbionts as a whole and not according to the nature of their relationship with hosts.

In the third part of the thesis, the diversification history of the highly specialized and host-specific feather mites is studied

- **Chapter 6** examines the relevance of cospeciation vs. host-shift speciation in highly specialized and host-specific symbionts
- **Chapter 7** examines the host specificity of vane-dwelling feather mites and the consequences of major host-switching for the diversification of highly specialized and host-specific symbionts.
- **Chapter 8** explores the eco-evolutionary scenario of host-shift speciation.

Chapter 1.

Global associations between birds and vane- dwelling feather mites

Understanding host–symbiont networks is a major question in evolutionary ecol- ogy. Birds host a great diversity of endo- and ectosymbiotic organisms, with feather mites (Arachnida: Acariformes: Analgoidea, Pterolichoidea) being among the most diverse of avian symbionts. A global approach to the ecology and evolution of bird–feather- mite associations has been hampered because of the absence of a centralized data repository. Here we present the most extensive data set of associations between feather mites and birds. Data include 12 036 records of 1887 feather mite species located on the flight feathers of 2234 bird species from 147 countries. Feather mites typically located inside quills, on the skin, or on downy body feathers are not included. Data were extracted from 493 published sources dating from 1882 to 2015. Data exploration shows that although most continents and bird families are represented, most bird species remain unexplored for feather mites. Nevertheless, this is the most comprehensive data set available for enabling global macroecological analyses of feather mites and their hosts, such as ecological network analyses. This metadata file outlines the structure of these data and provides primary references for all records used.

Published in: *Ecology*, 97, 3242.

Metadata

CLASS I. DATA SET DESCRIPTORS

A. Data set identity:

Title: Global associations between birds and vane-dwelling feather mites

B. Data set and metadata identification codes:

Suggested Data Set Identity Codes: global_fmbird.csv

C. Data set description

Originators: Heather Proctor started the compilation of the dataset for taxonomic purposes. The dataset was updated by Jorge Doña thanks to literature provided by Heather Proctor and Sergey Mironov within a project (CGL2011-24466) led by Roger Jovani about the evolutionary ecology of bird-feather mite associations. Then, Sergey Mironov checked the feather mite taxonomy and categorised the quality of the host-mite records and mite taxonomy. Jorge Doña, David Serrano and Roger Jovani checked the bird taxonomy and refined the whole dataset for consistency and data usability.

Abstract:

Understanding host-symbiont networks is a major question in evolutionary ecology. Birds host a great diversity of endo- and ectosymbiotic organisms, with feather mites (Arachnida: Acariformes: Analgoidea, Pterolichoidea) being among the most diverse of avian symbionts. A global approach to the ecology and evolution of bird-feather mite associations has been hampered because of the absence of a centralized data repository. Here we present the most extensive dataset of associations between feather mites and birds. Data include 12,036 records of 1887 feather mite species located on the flight feathers of 2234 bird species from 147 countries. Feather mites typically located inside quills, on the skin, or on downy body feathers are not included. Data were extracted from 493 published sources dating from 1882 to 2015. Data exploration shows that although most continents and bird families are represented, most bird species remain unexplored for feather mites. Nevertheless, this is the most comprehensive dataset available for enabling global

macroecological analyses of feather mites and their hosts, such as ecological network analyses. This metadata file outlines the structure of these data and provides primary references for all records used.

D. Key words: *Acari; birds; ectoparasites; feather mites; global; host-symbiont; long-term data; macroecology*.

CLASS II. RESEARCH ORIGIN DESCRIPTORS

A. Overall project description

Identity: The ecology and evolution of bird feather mite associations.

Originators: Same authors' contribution as above.

Period of Study: 2002–indefinite.

Objectives: To characterize bird-feather mite association networks to disentangle the evolutionary ecology and biogeography of these host-symbiont relationships.

Abstract: Same as above. These data are not part of a larger program of study.

Source(s) of funding: Ministry of Economy and Competitiveness; research projects CGL2011-24466 and CGL2015-69650-P, Severo Ochoa predoctoral grant to Jorge Doña (SVP-2013-067939) and Ramón y Cajal research contract to Roger Jovani (RYC-2009-03967) founded by the Spanish Ministerio de Economía y Competitividad and the Spanish Severo Ochoa Program (SEV-2012-0262). Several sequential Natural Sciences and Engineering Research Council of Canada Discovery Grants to Heather Proctor helped support construction of the original database. Checking of mite taxonomy and evaluation of records quality by Sergey Mironov was made under support by Russian Science Foundation (RSF No. 14-14-00621).

B. Specific subproject description

Site description: Data were collected from literature. Records in the dataset span 147 countries (see Fig. 3 for more details).

Experimental/Sampling design: All data were obtained from literature written mainly by feather mite taxonomists and ecologists from 1882 to 2015.

Research Methods: We explored all the relevant literature about associations between birds and those feather mite species inhabiting the surface of flight feathers (see References section). Most studies are primary taxonomic works (descriptions of species and higher taxa, revisionary studies) with a smaller subset being surveys of avian symbionts or studies of ecological relationships. Feather mites were collected from living or dead birds, with many of the post-mortem collections being performed on specimens held in ornithological collections of museums and universities. In the great majority of studies, feather mites were identified with the aid of a compound microscope after having been slide-mounted. In four studies, DNA barcodes complement morphological identifications.

Feather mite taxonomy reported here has been reviewed based on all the accessible world literature on feather mites. The world catalogue of this literature from the early studies of the 19th century until the middle of 1990s was compiled by Gaud and Atyeo (1996); publications from the mid-1990's until the present have been accumulated by H. C. Proctor and S. V. Mironov. All species names of feather mites in the present dataset are given according to the latest taxonomic reviews and revisions of corresponding genera or families. The full set of references used is provided below under the Supplemental descriptors.

Taxonomy used herein follows Gill and Donsker (2015) for birds, OConnor (2009) for feather mites at the familial and superfamilial level and Gaud and Atyeo (1996) at the generic level except for genera described after 1996. Country names have been tested to match with names available in the *countrySynonyms* function of the *R* package *rworldmap* version 1.3-1 (South, 2011).

CLASS III. DATA SET STATUS AND ACCESSIBILITY

A. Status

Latest update: 17 May April 2016 (bird taxonomic revisions added, some names updated; no new data added).

Latest Archive date: 17 May 2016.

Metadata status: 17 May 2016, metadata are current.

Data verification: All data was evaluated and triple-checked for accuracy.

B. Accessibility

Storage location and medium: The original data file is backed up in the JovaniLab linux server (www.jovanilab.com). In addition, it is available at The Ecological Society of America's Ecological Archives (http://esapubs.org/archive/).

Contact person: Jovani, Roger. Department of Evolutionary Ecology. Estación Biológica de Doñana (CSIC), Avda. Americo Vespucio, s/n, 41092, Isla de la Cartuja, Sevilla, Spain.

E-mail: jovani@ebd.csic.es

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Proprietary restrictions: This database is under an embargo for six months after the publication date for research purposes. After that, the dataset will be under CC BY 4.0 license, and usage of the data set must be acknowledged using the below citation.

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CLASS IV. DATA STRUCTURAL DESCRIPTORS

A. Data Set File

Identity: global_fmbird.csv

Size: 12,036 records, excluding header row.

Format and storage mode: Unicode (UTF-8), tab-delimited, no compression.

Alphanumeric attributes: Mixed.

Special characters/fields: None.

B. Variable definitions

Variable name	Variable definition	Storage type	Range numeric type

paper code	Reference source. Codes are linked to the global_fmbird_literature.csv file	numeric	1-650
mite_superfamily	Taxonomic superfamily of feather mites	Character	N/A
mite_family	Taxonomic family of feather mites	Character	N/A
mite_genus	Taxonomic genus of feather mites	Character	N/A
mite_species	Scientific name of feather mites (Genus, species)	Character	N/A
author_&_date	Feather mite species author citation	Alphanumeric	N/A
host_order	Taxonomic order of birds	Character	N/A
host_family	Taxonomic family of birds	Character	N/A
host_species	Scientific name of birds (Genus, species)	Character	N/A
host_ssp	Taxonomic subspecies of birds	Character	N/A
continent	Continent of the bird-mite sampling	Character	N/A
country_collected	Country of the bird-mite sampling	Character	N/A
locality	Detailed location of the sample	Alphanumeric	N/A
data_quality*	0 = Missidentification or Inquerenda; 1 = Contamination, Improvable or Probable association; 2 = High quality data (see Data limitation section for more details)	Numeric	0-2
quality_note	Notes about the data_quality values of the data. See Data limitations for details	Character	N/A

C. Data limitations.

*Data quality: Sergey Mironov has evaluated the quality of each record according to current taxonomic and ecological knowledge about bird-feather mite associations. This greatly increases the reliability of this dataset and hence its utility for other researchers. The main goal was to categorise the probability that a given record was informative of a natural association and not caused by methodological mistakes such as sample contaminations or misidentification, or by accidental mite transmission to bird species on which the mite species is very unlikely to maintain a persistent population. Taking into account the fact that the host specificity of feather mites is commonly very high, the following categories have been used:

- Code 2: Natural associations (11,336 records). Mite species recorded in their common bird hosts (i.e. a particular mite species has been repeteadly collected on a bird host).

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- Code 1 includes 694 records likely not natural associations. For 603 of these records it is specified the degree of certainty about the bird-feather mite association:

-- Probable association (168 records): the association has a moderate probability to be real. This category was used for records of mite species found on uncommonly examined host species, or on a host genus within the same host family as the common host(s) of a mite species.

-- Improbable (297 records): mite species recorded from a bird host belonging to another family than that to which the typical bird host belongs. In other words, it is likely a contamination, but it is possible that this is a real association. There are not many validated cases where a mite species is naturally distributed on hosts from several families.

-- Contamination (132 records): mite species was very likely found on a host because of an accidental contamination, in nature or in the course of collecting. This happens when a mite species was reported for a host from an order different than that to which the typical bird hosts belong (and the association has been not repeatedly reported in literature).

- Code 0 includes two categories:

-- Inquerenda (4 records). Species impossible to recognize and identify again, but the primary name was based on correct rules of the zoological nomenclature code.

-- Misidentification (2 records). Species misidentified in the original study.

Some of the records come from taxonomic reviews of previous literature. In consequence, some records could be duplicated in this dataset. Users should take this into account when using this dataset for their own studies.

The data cover a wide taxonomic and geographic range. However, the coverage is heterogeneous and most bird species and many geographic areas still remain unexplored for feather mites (Figs. 1-3).
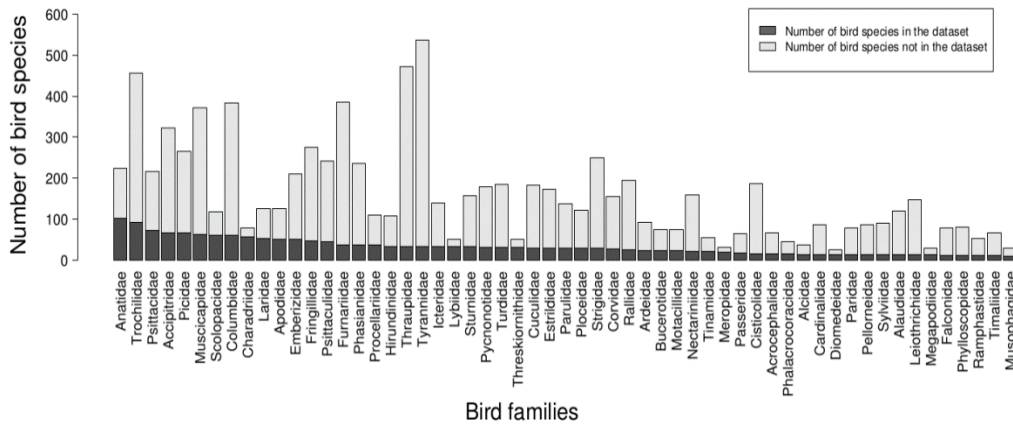
FIG 1. Number of bird species from each bird family included in the database (dark shading) and the number of unsampled species (light shading). Total number of species was taken from the IOC 5.4 list; see above for taxonomy references. Note that only families with more than six species in the database are included. Families are ordered from left to the right in a decreasing order according to the number of species studied.
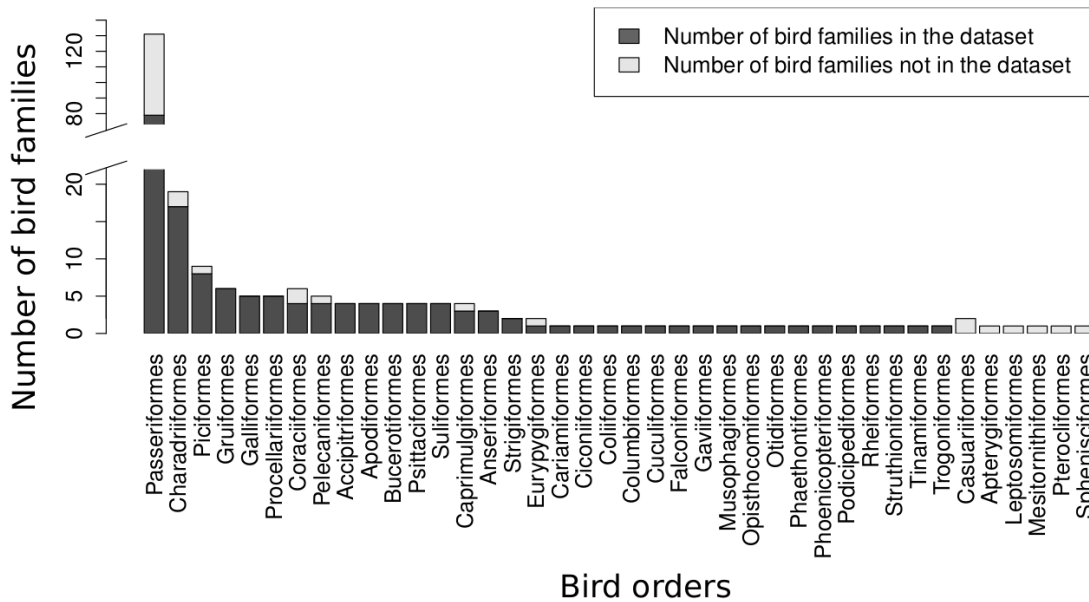

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Fɪɢ 2. Number of bird families from each bird order included in the database. The number of unsampled families was taken from the IOC 5.4 list; see above for taxonomy references. Orders are sorted in decreasing order from left to the right according to the number of families studied per order. Note that Sphenicisformes and Apterygiformes are included despite not being expected to harbour feather mites typical of flight feathers (the focus of the present dataset) because they do not have functional flight feathers.
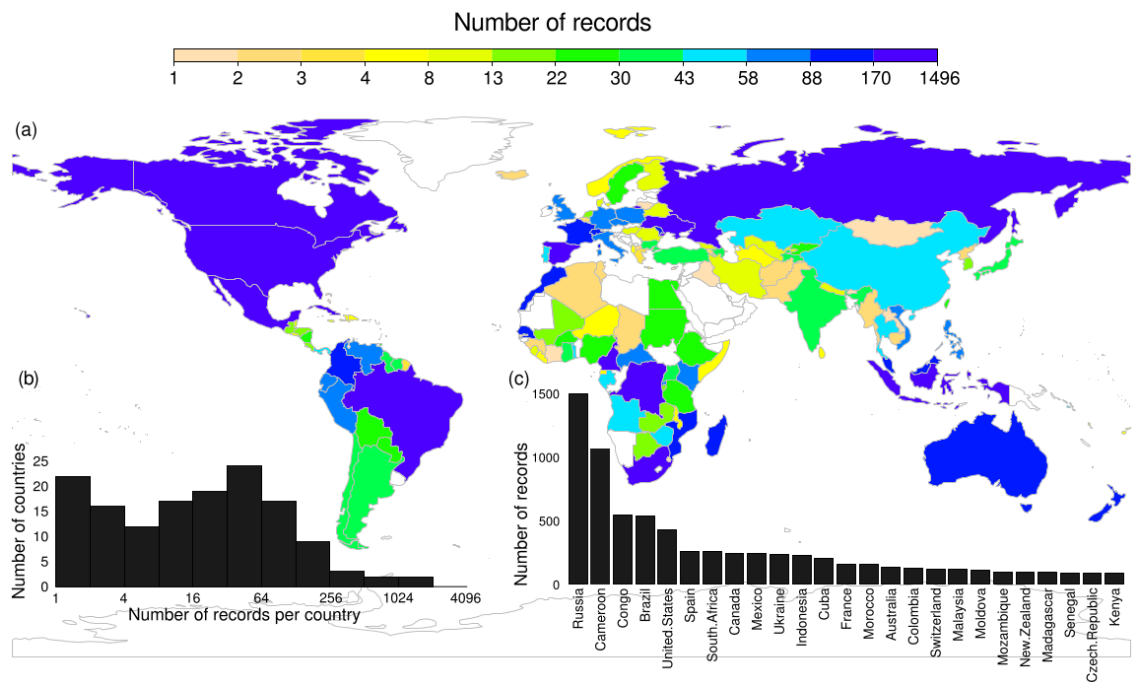

Fɪɢ 3. Geographic distribution of records. (a) Countries are coloured according to the number of records in a quantile scale. (b) Frequency distribution of the number of records per country. (c) Frequency distribution of the number of records for countries with more than 88 records.

Class V. SUPPLEMENTAL DESCRIPTORS

A. Data set references

Paper code	Reference

1	McClure, H. E., Ratanaworabhan, N., Emerson, K. C., and Atyeo, W. T. 1973. Some ectoparasites of the birds of Asia. 1-219
2	Atyeo, W. T., and Peterson, P. C. 1976. The species of the feather mite family Recttijanuidae (Acarina: Analgoidea). Journal of the Georgia Entomological Society 2: 349-366
3	Atyeo, W. T., and Pérez, T. M. 1990. Feather mites of the *Aralichus canestrinii* (Trouessart) complex (Acarina, Pterolichidae) from New World parrots (Psittacidae). II. From the genera *Aratinga* Spix, *Deroptyus* Wagler, *Leptosittaca* Berlepsch and Stolzmann, *Ognorhynchus* Bonaprte, *Pionites* Heine, and *Pyrrhure* Bonaparte, and conclusions to the study. Fieldiana 62: 1-30
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17	Atyeo, W. T., and Pérez, T. M. 1988. Species in the genus *Rhytidelasma* Gaud (Acarina: Pterolichidae) from the Green Conure, *Aratinga holochlora* (Sclater) (Aves: Psittacidae). Systematic Parasitology 11: 85-96
18	Atyeo, W. T., and Gaud, J. 1991. *Lorilichus*, a new genus of feather mites (Acarina, Pterolichidae) from lories and lorikeets (Aves, Psittacidae). Systematic Parasitology 18: 139-146
19	Atyeo, W. T. 1989. *Aralichus porrectus* (Mégnin and Trouessart) and related feather mite species (Acarina, Pterolichidae) from parrots of the genus *Brotogeris* Vigors (Aves, Psittacidae). Systematic Parasitology 14: 101-111
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Chapter 2.

DNA barcoding and minibarcoding as a powerful tool for feather mite studies.

Abstract

Feather mites (Astigmata: Analgoidea and Pterolichoidea) are among the most abundant and commonly occurring bird ectosymbionts. Basic questions on the ecology and evolution of feather mites remain unanswered because feather mite species identification is often only possible for adult males, and it is laborious even for specialized taxonomists, thus precluding large-scale identifications. Here, we tested DNA barcoding as a useful molecular tool to identify feather mites from passerine birds. Three hundred and sixty-one specimens of 72 species of feather mites from 68 species of European passerine birds from Russia and Spain were barcoded. The accuracy of barcoding and minibarcoding was tested. Moreover, threshold choice (a controversial issue in barcoding studies) was also explored in a new way, by calculating through simulations the effect of sampling effort (in species number and species composition) on threshold calculations. We found one 200-bp minibarcode region that showed the same accuracy as the full-length barcode (602 bp) and was surrounded by conserved regions potentially useful for group-specific degenerate primers. Species identification accuracy was perfect (100%) but decreased when singletons or species of the Proctophyllodes pinnatus group were included. In fact, barcoding confirmed previous taxonomic issues within the P. pinnatus group. Following an integrative taxonomy approach, we compared our barcode study with previous taxonomic knowledge on feather mites, discovering three new putative cryptic species and validating three previous morphologically different (but still undescribed) new species.

Introduction

Feather mites (Acari: Astigmata: Analgoidea and Pterolichoidea) are among the most abundant ectosymbionts living on birds (Gaud & Atyeo 1996; Proctor & Owens 2000; Proctor 2003). Among them, plumicolous mites are those living permanently on the feather surfaces of birds (Proctor 2003). In Europe alone, about 130 species of plumicolous feather mites (from 31 genera and 9 families) have been described on passerines, and a number of species have yet to be described (Mironov 1996; S. Mironov, personal observation).

Feather mites are present in almost all avian groups. However, there are many questions surrounding feather mite evolutionary ecology that remain unanswered (Proctor & Owens 2000; Proctor 2003). For example, it is still debated whether the nature of bird/feather mite relationships is parasitic, commensalistic or even mutu- alistic (Blanco et al. 2001; Figuerola et al. 2003; Galván et al. 2012). This controversy may stem from the fact that questions on feather mite ecology have been traditionally addressed by mainly correlating the combined abundance and prevalence of different mite species with host traits (e.g. Galván et al. 2012). However, we now know that the abundance of feather mites is not only shaped by host traits (e.g. body size, Galván et al. 2012; size of the uropygial gland, Galván & Sanz 2006), but also by the species composition of feather mites living on a bird (Fernández-González et al. 2013), or differently affecting feather mite species or even by environmental factors (Dubinin 1951; Fernández-González et al. 2013; Meléndez et al. 2014). Thus, it is clear that a greater knowledge of the feather mite community living on each bird species and on each bird individual would accelerate our understanding of the evolutionary ecology of this interaction.

This approach has rarely been addressed because feather mite species identification is a difficult task; females of some taxa and immature stages of many families are often indistinguishable, and even for males, accurate identification requires advanced taxonomic skills. Moreover, in some groups of closely related species (e.g. the pinnatus species group from the genus *Proctophyllodes*), it is extremely difficult to identify single individuals based on morphological characters (S. Mironov, personal observation). In this scenario, an accurate

molecular tool for species identification would be highly valuable. In similar ecological systems, these problems have been successfully addressed by combining morphological and DNA barcoding approaches (i.e. integrative taxonomy approach), which has also been proposed as a powerful framework for species discovery and identification (Besansky et al. 2003; Smith et al. 2006; Hajibabaei et al. 2007; Schlick-Steiner et al. 2010).

On the other hand, species identifications based on barcoding do not work equally well in all groups, thus requiring a prior test of effectiveness before application to specific taxa (Moritz & Cicero 2004; Virgilio et al. 2012; Collins & Cruickshank 2013). This test requires an extensive barcoding library, which is not available for feather mites where only a few species (c. 20 sp) have been barcoded (Ratnasingham & Hebert 2007; Dabert et al. 2008, 2011; Jinbo et al. 2011; Glowska et al. 2014). However, the efficacy of barcoding has never been tested for feather mites.

DNA barcoding is based on amplifying and sequencing DNA regions that are informative at the species level. For several animal groups, the mitochondrial 648-bp region of the cytochrome oxidase subunit 1 (COI) gene has been demonstrated as a useful barcode (Hebert et al. 2003a, b; Savolainen et al. 2005; Hajibabaei et al. 2007). It has also proven effective in complex scenarios, even revealing cryptic species (Hebert et al. 2004). Here, we provide the largest library of DNA barcodes currently available for feather mites covering the majority of European passerine species, and we test the accuracy of the method. Moreover, we explored other issues around barcoding of feather mites relevant to their extended usability and confidence in addressing issues of molecular systematics:

1   First, DNA barcodes of typical size (more than 600 bp) may be difficult to obtain with degraded DNA (e.g. museum specimens and dietary research) or may suffer technological restrictions. For instance, the more accurate and informative massive parallel sequencing technologies are currently limited to short DNA frag- ments. In these conditions, minibarcodes have proven to be very successful (Sundquist et al. 2007), so we identified potential minibarcodes for feather mites and explored their efficacy.

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2   The use of thresholds to differentiate species has been repeatedly discussed in the DNA barcoding literature, finding that no single threshold is optimal for all spe- cies (Puillandre et al. 2012; Virgilio et al. 2012; Collins & Cruickshank 2013). Moreover, the accuracy of a threshold-based approach critically depends upon the level of overlap between intra- and interspecific variation across a phylogeny (Meyer & Paulay 2005). Also, it is known that the overlap is considerably greater when a larger proportion of closely related taxa are included and that barcoding may perform poorly in incompletely sampled groups (Moritz & Cicero 2004; Ratnasingham & Hebert 2007). Therefore, here, we simulated the effect of library size (number of species) and species composition in the sample upon threshold calculation to test the robustness of our results against sampling issues.

3   Finally, we tested the congruence of the barcode library of feather mites presented here with the previous taxonomic studies of feather mites. For this pur- pose, we followed an integrative taxonomy approach where we combined morphological identifications, automated procedures for primary species delimita- tion (Automatic Barcode Gap Discovery, ABGD) and Bayesian phylogenetic analyses (Huelsenbeck et al. 2001; Puillandre et al. 2012).

Materials and methods

Sampling

Feather mite specimens were collected during 2011–2013 from live birds captured with mist nets in different localities of Spain and Russia (Table S1, Supporting information). Mites were manually collected from the feathers using a flattened preparation needle or a cotton swab impregnated with ethanol and preserved at -20 °C in tubes with 96% ethanol. When possible, mite samples were taken from different geographical populations and from different host species, and one to five individuals from each putative mite species were sequenced (see below). After DNA isolation, mites were mounted on slides in Faure's medium according to standard techniques for small mites (Krantz & Walter 2009) and then identified by S.M. under

a Zeiss AX10 light microscope. A total of 361 specimens were identified based on morphological characters according to world revisions of the genera *Proctophyllodes* (Atyeo & Braasch 1966) and *Trouessartia* (Santana 1976) and other corresponding tax- onomic publications. The genus *Proctophyllodes* is the most species-rich genus (161 species) among feather mites, and the above-mentioned controversial pinnatus group is the most speciose within the genus, currently including 37 species (Mironov et al. 2012). Mites of this group are very uniform morphologically and differentiation of closely related species is mainly based on male characteristics. As morphological overlaps between species of this group have never been specifically studied, identification of species based on single specimens is often difficult. In this context, it is also possible that phylogenetically distant avian species described as hosts of, presumably, the same mite species actually harbour separate cryptic species. All mounted specimens were preserved at the Estación Biológica de Doñana (Spanish National Research Council, CSIC, Seville, Spain) with accession nos (EBD1201ART–EBD1561ART).

DNA isolation, amplification and sequencing

Genomic DNA was extracted using HotSHOT (Truett   et al. 2000). After extraction, exoskeletons were separated from the extraction volume and stored in 96% etha- nol. A segment of approximately 650 bp of the COI region was amplified by PCR with degenerate primers bcdF05 (50-TTTTCTACHAAYCATAAAGATATTGC-30) and   bcdR04 (50-TATAAACYTCDGGATGNCCAAAAAA-30) (Dabert et al. 2008). PCRs were carried out in 20 µl reaction volumes containing 1x $(NH_4)_2SO_4$ reaction buffer (Bioline), 2.5 mM MgCl2, 1x BSA, 0.25 mM DNTPs, 2 µm of each primer, 1.25 U BIOTAQ$_{TM}$ (Bioline) and 7 µl of DNA template. The reaction followed a touchdown PCR profile: 95 °C for 3 min, 20 cycles of 95 °C for 1 min, 55 °C for 30 s with a decrease of 0.5 °C every cycle, 72 °C for 1 min, and 20 cycles of 95 °C for 1 min, 45 °C for 30 s and 72 °C for 1 min, with a final extension step of 72 °C for 5 min. PCR products were quantitatively assessed by electrophoresis on a 2% agarose gel,

and visible bands corresponding to the COI fragment size were sequenced in two directions. COI sequencing was carried out using the Sanger method and performed by Macrogen, Europe (Holland) and by Molecular Ecology Lab at the Estación Biológica de Doñana with bcdF05 and bcdR04 (Dabert et al. 2008).

Data analysis

<u>Sequence editing and phylogenetic analyses.</u> The forward and reverse DNA sequences were edited and manually trimmed to 602 bp using SEQUENCHER 5.2 software. Sequences were aligned using CLUSTALW with default settings (Larkin et al. 2007) in Geneious (Drummond et al. 2009) and deposited in GenBank with the accession nos KP193464-KP193819. The final alignment was visually revised using MEGA (Tamura et al. 2013) and comprised 362 sequences including Freyana anatina (GenBank acc. no. GQ864352), as an outgroup taxon. JMODELTEST 2 (Darriba et al. 2012) was used to determine the appropriate model of sequence evolution for Bayesian analyses. Mr BAYES version 3.2 (Ronquist et al. 2012) was used to run two parallel analyses each with GTR + G + I as the model of evolution, each consisting of four Markov chains of 4 000 000 generations. Conver- gence of each analysis was evaluated using TRACER 1.4.1 (Rambaut & Drummond 2007) to check that ESS values were all >200 (default burn-in).

<u>Barcoding analysis.</u> Assessing specimen identification success. To assess barcoding accuracy in specimen identification, we used the genetic distances based on the 'best close match' (BCM) method presented by Meier et al. (2006). For the analyses, we used the bestCloseMatch function of the R package SPIDER version 1.3–0 (http://spider.r-forge.r-project.org/) (Brown et al. 2012). BCM reports four different identification categories: (i) 'correct' when the name of the closest match is the same than the specimen considered; (ii) 'incorrect' when the name of the closest match is different than the specimen considered; (iii) 'ambiguous' when more than one species is the closest match; and (iv) 'no id' when no

species is found within the given threshold. Thus, we obtained a metric of identification success calculated as the percentage of correct identifications. Following Collins et al. (2012), we considered singletons as a different identification scenario where the only possible identification result is 'incorrect' or 'no id'. Therefore, we reported results with singleton species included and excluded. Finally, we also evaluated the performance of barcode sequences in species identification conducting a barcode gap analysis in BOLD (Ratnasingham & Hebert 2007).

Checking threshold confidence. For threshold calculations, the local minima function of the *R* package *SPIDER* was used. It is based on the concept of the barcoding gap, where a dip in the density of genetic distances indicates the transition between intra- and interspecific distances. As the identity of the species composition of the library may affect the threshold calculated, we explored whether and how our calculated threshold stabilized across a simulated increasing sample of species from our available library. To do so, for each possible sample size from 1 to 72 (the number of species in our library), we created 1,000 random combinations of different species and calculated (with local minima function) the threshold for each subsample. Moreover, following Collins et al. (2012), we evaluated a range of threshold values for their effect on both the false-positive (a) and false-negative (b) error rates using threshold optimization function in the *SPIDER* package. The optimum threshold was defined where cumulative errors were minimized.

Minibarcodes. The *sliding window function* slide analyses in *SPIDER* (Brown et al. 2012) was used to determine the shortest informative window best discriminating the feather mite sequences of reference. This function extracts all possible windows (DNA sequences) of a chosen size in a DNA alignment and performs, for each window, distance measures including the following: (i) proportion of zero nonconspecific distances; (ii) number of diagnostic nucleotides; (iii) number of zero-length distances and overall mean distance; (iv) tree-based measures including the proportion of species that are monophyletic; and (v) the proportion

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of clades that are identical between the neighbour-joining tree calculated for the window and the tree calculated for the full data set.

After this, the shortest informative window was selected by considering (following Boyer et al. 2012) the proportion of zero pairwise nonconspecific distances in the matrix, and the proportion of identical clades shared between the neighbour-joining tree derived from the full 602-bp data set (and those derived from each window). Windows with no zero nonconspecific distances and a proportion of identical clades >85% for shallow nodes (i.e. nodes tipwards of the median node depth) were considered as highly informative because they allow accurate specimen identification and provide a good representation of the tree topology for the full data set. Windows of 50, 100, 150 and 200 bp were analysed and compared to determine the shortest highly informative window. Then, identification success of each of the four most informative selected windows was also tested by BCM as was performed before for total length barcode. Tentative regions for group-specific degenerate primers were explored for the selected minibarcode, using nucleotide diversity analyses conducted on *DNASP* software (Librado & Rozas 2009).

Primary species delimitation. The ABGD method (Puillandre et al. 2012) was used with phylogenetic analyses to review the primary species discovery in our groups. This method uses many prior thresholds to propose partitioning of specimens into primary species hypotheses (PSHs) based on the distribution of pairwise genetic distances. In this distribution of pairwise differences between sequences, a gap exists between intraspecific and interspecific diversity. This 'barcode gap' can be used as a threshold for delimiting primary species under the consideration that individuals within species are more similar than those between species. The COI sequence alignment was used to compute matrices of pairwise distances using the Kimura-2-parameter (K2P) models with *sppDistMatrix* function in *SPIDER* (Brown et al. 2012). Matrices were then used as inputs on the ABGD webpage (http://wwwabi.snv.jussieu.fr/public/abgd/abgd- web.html), using the default settings search on a set of prior minimum genetic distances ranging from

0.001 to 0.1. Lastly, ABGD output was visually compared with Bayesian phylogeny to check for congruence.

Additionally, we used the Refined Single Linkage (RESL) algorithm of BINs, which performs an initial analysis using a 2.2% sequence divergence as the minimum distance between clusters (Ratnasingham & Hebert 2013). BINs splits were also visually compared with ABGD partitions and Bayesian phylogeny to check for congruence.

Results

A total of 361 individual mites from 72 species and 12 genera were identified by morphology under the microscope, and their mitochondrial COI region was subse- quently sequenced. All nucleotides were translated into functional protein sequences in the correct reading frame, with no stop codons or indels observed in the data. Each species was represented by five individuals on average; 20 species (27.3%) had only one individual (i.e. singletons; see other sample statistics in Table 1).

Table 1 Summary of descriptive barcode statistics for feather mite data analysed

Individuals	361
Species	72
Mean individuals per sp. (range)	5 (1–22)
Singletons	20
Genera	12
Seq. length (bp)	602
Number of haplotypes	319
Haplotype gene diversity	0.998
Mean intraspecific distances (range, SD)	2% (0–11, 2.04)
Mean smallest interspecific distances (range, SD)	9% (0–22, 4.83)

Identification success rates using DNA barcodes

Using BCM, identification success was usually high (>88%) when singletons were excluded and perfect when both the pinnatus group and the singletons were excluded. 'ambiguous'

identifications increased mainly when the pinnatus group was included in the analyses (Table 2). The same pattern was observed when the bar- coding gap analysis in BOLD was used. All species from the pinnatus group always presented nearest neighbour values smaller than the corresponding maximum intra- specific distances. Singleton species always resulted in nearest neighbour distances above the threshold (3.42%, see below), thus reporting 'no id' in the analyses.

Table 2 Percentage of the different categories (see Materials and methods) of identification success for best close match with different combinations of singletons and pinnatus group included or excluded. The number of specimens used is shown (*n*)

Singletons	*pinnatus* group	Correct	Incorrect	Ambiguous	No id	*n*
Included	Included	83	3	8	6	361
Excluded	Included	88	4	8	0	342
Included	Excluded	93	0	0	7	300
Excluded	Excluded	100	0	0	0	281

Threshold confidence and accuracy

We obtained a threshold value of 3.42%, which remained the same after threshold optimization (Fig. 1). Our simulations (see 'Checking threshold confidence') showed that the threshold stabilized at around 30 mite species, well before reaching the 72 species of our whole data set, thus suggesting that additional sampling would not significantly change the threshold for feather mites of European passerine birds (Fig. 1).
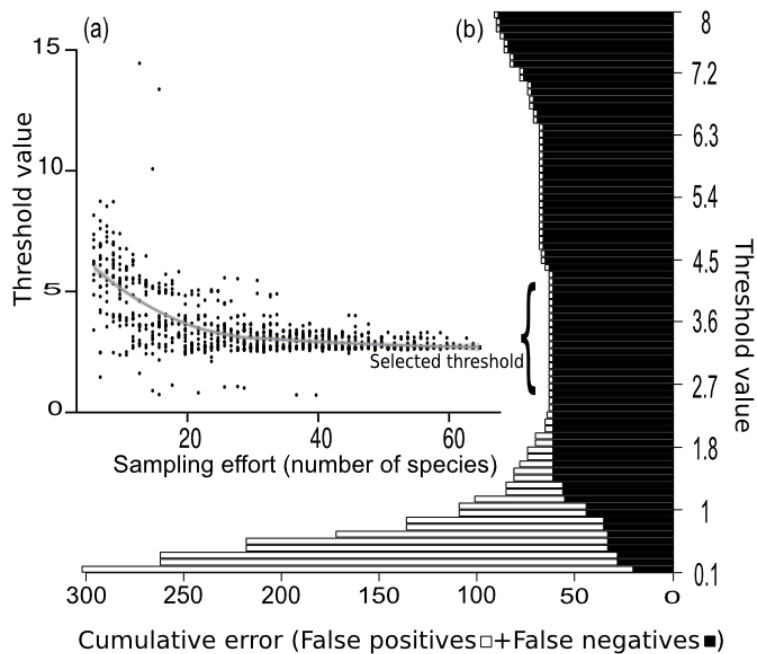
Fig. 1 Selected threshold after checking confidence in two ways. (a) Taxon sampling effect upon threshold calculation. Loess fit line with confidence limits is shown. (b) Shows cumulative error and threshold optimization. Error rates summed across a range of distance thresholds from 0, 1–8% in 0.1% increments.

Minibarcodes

Sliding window analyses revealed short informative regions from 50 to 200 bp (Table 3). For the four differently sized windows (one per window length), the proportion of zero pairwise nonconspecific distances was 0. Therefore, the criteria with which to choose the best windows were the proportion of identical clades shared between the neighbour-joining tree derived from the full-length data set and those derived from each window. After BCM analyses of all sized best windows, a 200-bp window (located from 295 to 495 bp in our alignment) was the only minibarcode to obtain exactly the same identification success as the total length barcode. Moreover, this region was surrounded by conserved regions (Fig. 2), thus being potentially useful to design group-specific degenerate primers.
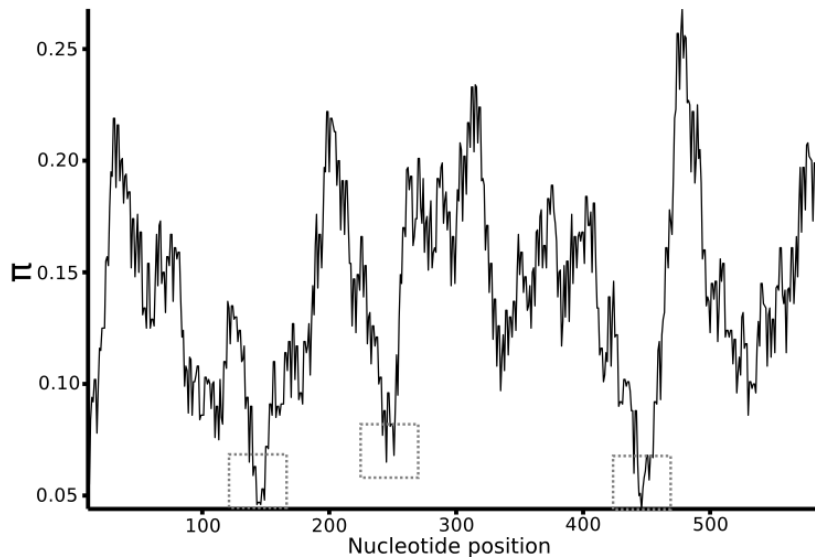
Fig. 2 Distribution of substitutions, measured as nucleotide diversity (π), in the alignment. Window length = 20 sites. Dashed squares represent low nucleotide diversity regions. Note that the central low diversity region is close to the starting position of the best windows shown in Table 3.

Integrative taxonomy

DNA barcoding was robust when comparing feather mites of the same species sampled at distant locations (Russia vs. Spain) or the same mite species from dif- ferent bird hosts (Fig. 3). However, our phylogenetic, RESL and ABGD analyses showed a strong genetic structure of two clusters within three *Proctophyllodes* species: *P. musicus*, *P. stylifer* and *P. clavatus*. In two of these species, clusters within mite species occurred in different but closely related bird species: *Turdus merula* and *Turdus philomelos* on *P. musicus* (Figs 3, 4a and S1, Supporting information), and *Parus major* and *Cyanistes caeruleus* on *P. stylifer* (Figs 3 and S1, Supporting information). A similar situation occurred in *P. clavatus*, with a cluster with a single individual found on *Acrocephalus schoenobaenus*, while the rest of the *P. clavatus* were found on *Sylvia borin*. In this case, the individual on *A. schoenobaenus* was even closer to *Proctophyllodes cetti* than to the other *P. clavatus* (Figs 3 and S1,

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Supporting information). In all three cases, evidence thus suggests that these may be morphologically cryptic mite species living on different (but closely related) bird hosts.

Moreover, our phylogenetic analyses support the hypothesis that three previously undescribed mite species, recognized by morphology (S. Mironov, personal observation), do belong to distinct species, because they show well-isolated clusters in our phylogeny. Two species (from the genera *Proctophyllodes* and *Mesalgoides*) were from the red crossbill, *Loxia curvirostra*, and one from the genus *Dolichodectes* was hosted on the melodious warbler *Hippolais polyglotta* (Figs 4b and S1, Supporting information).
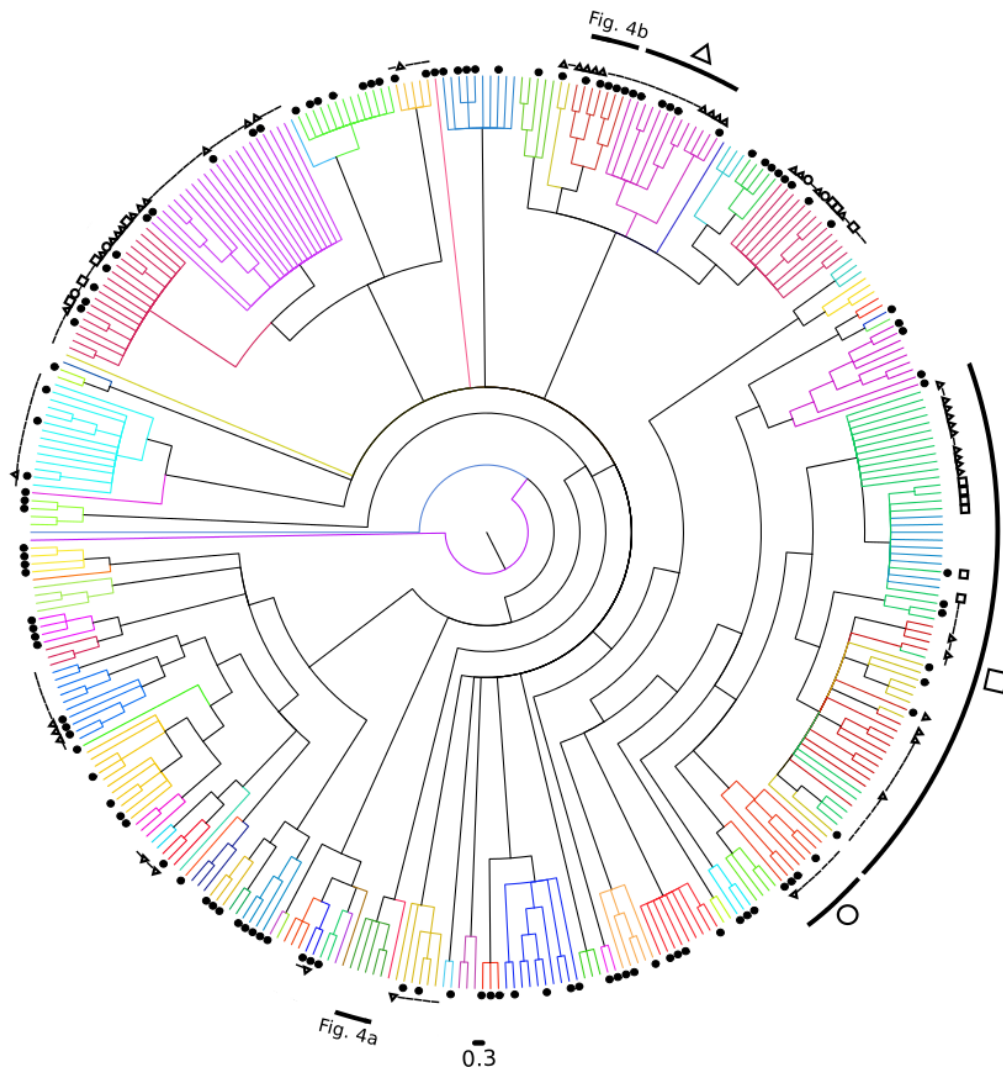
Fig. 3 Bayesian phylogeny for the 361 individual mites of the 72 feather mite species in this study based on cytochrome oxidase subunit 1. Each colour represents a different species according to morphological identification. The large square shows the pinnatus group, where different species occur within the same clusters; this does not occur in the rest of the phylogeny (despite that similar colours may suggest so). The large circle and triangle indicate *Proctophyllodes clavatus* and *P. stylifer*, respectively. Filled dots represent individuals from Russia. Small symbols show bird species identity (same arbitrary host symbols are used for different mite species) when a species of mite was sampled in more than one bird species (when a mite species was found in a single bird species, no symbol was used).

Discussion

Here, we found a high identification success (100% without singletons and the conflictive pinnatus group) using BCM (Meier et al. 2006) for our feather mite library, as previously reported in other arthropod barcoding studies (Virgilio et al. 2010). Contrary to other DNA barcoding studies, in which COI showed high genetic structure between populations within species (Tavares et al. 2011), our results showed high robustness with no geographical genetic structure for our marker, despite the fact that we sequenced the same feather mite species from distant populations of European passerines and the same mite species inhabiting different bird hosts. Previous studies using COI in taxonomical studies of particular feather mite species have reported low intraspecific and higher interspecific genetic distances (Dabert et al. 2008, 2011; Jinbo et al. 2011; Glowska et al. 2014) suggesting its usefulness for species identification. Here, we extend current information providing the largest library of barcodes for feather mites, and our analyses of this library confirm that the COI region is useful for species identification in this group.

Most of the current popular massive parallel sequencing tools (Illumina, Ion Torrent, etc.) have important benefits but also some constraints, such as the limited length of the sequences (Mardis 2011). In this context, minibarcodes have been presented as a good option for specimen identification in DNA barcoding (Meusnier et al. 2008). In this work, as reported for fish and butterflies (Hajibabaei et al. 2006), we obtained the same identification success with a short region of 200 bp and present it as a tentative minibarcode region for feather mites. Thus, at least for feather mite species identification, minibarcodes may be a useful tool.

Choosing appropriate thresholds that can separate species is one of the main challenges and concerns for DNA barcoding studies (Ferguson 2002). This is the basis of important criticisms of barcoding methods, which state that single-gene thresholds for species discovery can result in substantial errors in detecting new species with recent divergence times. Our innovative approach to the assessment of the threshold stability within a barcoding library may help discern when a threshold is usable for a certain group. It may

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be considered that the early stabilization confers a measure of confidence in the calculated threshold for a particular sampled group. In our library, we achieved a high threshold stabilization at a level of 30 species (<50% of total library). Moreover, species composition had a small impact on the final threshold obtained. This threshold was 3.14%, interestingly close to the 3% commonly used in barcoding literature (Hebert et al. 2003a, b). Nevertheless, it is important to note that for threshold calculations, we used the *local minima* function of the *R* package *SPIDER* (Brown et al. 2012). This is based on the concept of the barcoding gap, which has been proven to be very effective in some groups (as reported here for feather mites) but not in others (Čandek & Kuntner 2014). Therefore, these simulations may be sensitive to the same benefits (easy to calculate, easy to interpret and very repeatable among different groups) and problems (mainly overlaps between intra- and interdistances in some groups) as the barcoding gap approach (Wiemers & Fiedler 2007; Čandek & Kuntner 2014).

The pinnatus group is composed of species highly similar in morphology and is the most diverse species group in the *Proctophyllodes* genus (Atyeo & Braasch 1966), thus suggesting a recent and rapid diversification. Our analyses confirmed previous suspicions of taxonomic issues within this group, thus encouraging further additions of new markers and integrated taxonomic approaches, likely leading to a reconsideration of current taxonomic descriptions and hopefully identification improvements thanks to a multilocus barcoding approach (Dupuis et al. 2012).

The tree inferred from barcoding data (Fig. S1, Supporting information) confirmed most of the taxonomies of the relationships of the investigated taxa. The barcoding served as most precise tool for revealing relationships of feather mites at specific and generic levels. This method allowed the clear differentiation of most mite species. It is important to note that these data revealed the (genetic) homogeneity of a mite population of a particular species associated with a particular passerine species within the limits of Europe. On the other hand, these data allowed the detection of supposedly cryptic species inhabiting different hosts in the same territory.

With respect to species discovery, we also used an integrative taxonomy approach, with a single-gene analysis from 'DNA barcoding' and a morphological study to determine species hypotheses (Schlick-Steiner et al. 2010). The single-gene data set was analysed with bioinformatic species delimitation tools, such as ABGD or RESL and contrasted with phylogenetic trees (Puillandre et al. 2012; Ratnasingham & Hebert 2013; Roy et al. 2014). This was useful to confirm the existence of three undescribed species and to discover three likely cases of cryptic species within three morphologically recognized *Proctophyllodes* species (*P. musicus*, *P. stylifer* and *P. clavatus*), each associated with a pair of closely related host species. Interestingly, one of these cases (*P. stylifer*) was also reported in an independent study by Dabert et al. (2005), thus giving further support to the hypothesis that *P. stylifer* may be composed of at least two cryptic species. In *P. clavatus*, a cluster with the single mite individual sampled from *Acrocephalus shoenobaenus* is clearly distant from the rest of the *P. clavatus* mites sampled from *S. borin* hosts, but is distinctly closer to *P. cetti* sequences. *P. clavatus* and *P. cetti* show very similar morphology. Association of *P. clavatus* with *A. schoenobaenus* is not accidental, as it was previously recorded by other authors (Atyeo & Braasch 1966). All these cases of potentially cryptic species require further study.
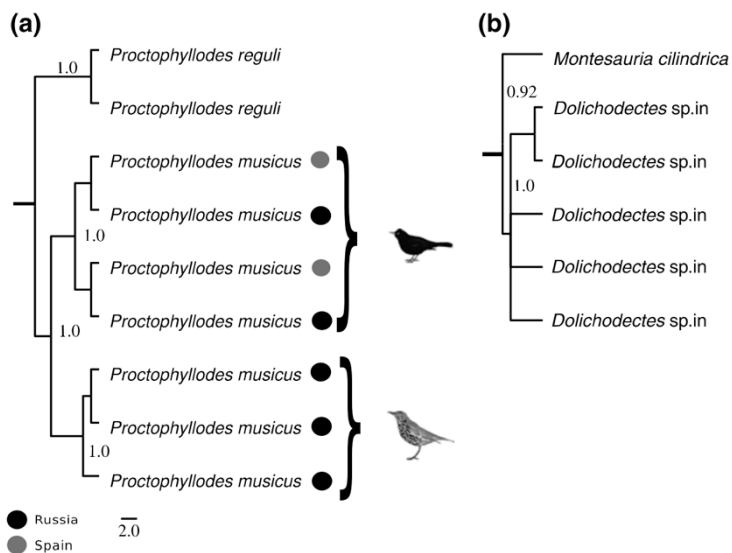
Fig. 4 Two examples of insights from the integrative taxonomic approach (see Fig. 3, for the relative position of these examples within the larger phylogeny). (a) Tentative cryptic species from Proctophyllodes musicus sampled from close bird species: black- birds (Turdus merula), top; and song thrushes (Turdus philomelos), bottom. (b) Confirmation of a tentative new species of the genus Dolichodectes previously identified as such by S.M. by morphology. Posterior probabilities values above 0.75 are shown.

Chapter 3.

Enabling large-scale feather mite studies: An Illumina DNA metabarcoding pipeline

Abstract

Feather mites are one of the most common and diverse ectosymbionts of birds, yet basic questions such as the nature of their relationship remains largely unknown. A reason for feather mites being understudied is that their morphological identification is often virtually impossible when using female or young individuals. Even for adult male specimens this task is tedious and requires advanced taxonomic expertise, thus hampering large-scale studies. In addition, molecular-based methods are challenging because of the low DNA amounts usually obtained from these tiny mites do not reach those required for high-throughput sequencing. This work aims to overcome these issues by using a DNA metabarcoding approach to accurately identify and quantify the feather mite species present in a sample. We present a high-throughput method for feather mites' identification using a fragment of the COI as marker and the Illumina Miseq technology. We tested this method by performing three different experiments over a total of 11,861 individual mites (5,360 morphologically identified). First, we tested the probability of detecting a single feather mite specimen in a heterogeneous pool of individuals. Second, we studied the relationship between the proportion of individuals in a sample to that of the sequences retrieved in a set of different species combinations. Third, we tested the efficacy of the degenerate primers and investigated the relationship between the number of mismatches and PCR success. Finally, we applied our DNA metabarcoding pipeline to a total of 6,501 unidentified and unsorted feather mite individuals sampled from 380 European passerine birds, belonging to 10 different bird species. Our results show that this proposed pipeline is suitable for the correct identification and quantitative estimation of the relative abundance of feather mite species in complex samples, especially when dealing with a moderate number (>30) of individuals per sample.

Introduction

Symbionts comprise a major component of Earth's biodiversity (Dobson et al. 2008; Poulin 2014; Tripp et al. 2017) and are central to ecosystem functioning. For instance, parasites are responsible for more than 75% of the links in natural food webs (Lafferty et al. 2006). Most symbiont species remain, however, poorly understood in comparison to free-living organisms (Duarte Rocha et al. 2016; Tripp et al. 2017). An important reason are the challenges for their study. For identification purposes only, many symbionts present morphological characters that are inconspicuous or insufficient for identification. In addition, DNA-based methods face challenges as well, such as the usual low DNA amount obtained from tiny symbionts that does not fit the minimum required for high-throughput sequencing (e.g. Allen et al. 2017). Overall, these and other methodological constraints have hampered the existence of suitable datasets of symbiont species, thus precluding research on many topics. For example, in 2004 the first predictions on which free-living species will commit extinction due to global change were published, while until the present year (i.e. 13 years later) there were not enough data for this kind of studies on symbiont species (Thomas et al. 2004; Carlson et al. 2017).

Environmental DNA (eDNA), DNA metabarcoding and mitochondrial metagenomics are useful methods for ecological and biodiversity studies which allow the identification of virtually all the biodiversity present in a bulk sample by comparing the sequences obtained against a reference database of previously identified organisms (Taberlet et al. 2012A, b; Gómez-Rodríguez et al. 2015). In addition, these methods further allow retrieving useful genetic information (Gómez-Rodríguez et al. 2016; Elbrecht et al. 2017b). DNA metabarcoding (Taberlet et al. 2012a) has rapidly become widely used, as it enables rapid and cost-effective molecular identifications of bulk samples when organisms are hard or impossible to individualise (Taberlet et al. 2012a; Soininen et al. 2013; Jousselin et al. 2016). In this way, it has become of crucial importance when routinely studying and monitoring microorganism communities, such as bacteria (e.g. De Tender et al. 2015; Reva et al. 2015), archaea (e.g. Navarro-Noya et al. 2015), unicellular fungi (e.g. Reva et al. 2015), or protists (e.g. Geisen et al. 2015). The method is based on the mass PCR amplification, using universal primers, of a given genomic region which varies among different groups of organisms. For animals, the genomic region of choice has historically been a fragment of the mitochondrial gene that codes for the cytochrome oxidase subunit I (COI) (even though it is not the best

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choice in some situations, Deagle et al. 2014) or a fragment of the nuclear 18S gene (Hebert et al. 2003; Deagle et al. 2014). DNA metabarcoding approaches take advantage of high throughput sequencing (HTS) technologies, which are becoming ever cheaper and more accessible (Stephens et al. 2015). However, despite the virtually universal applicability of these methods, each organismal group has its own particularities that can limit its application (e.g. Arribas et al. 2016; Linard et al. 2016; Pornon et al. 2016; Elbrecht et al. 2017a). This is especially true for symbiont organisms, because they are largely understudied, compared to free-living organisms, and therefore reference databases are far less comprehensive (Baker et al. 2016). Also, due to the small nature of many of these symbionts, it is often difficult to obtain large quantities of DNA, which complicates the molecular work, by making PCRs less efficient and by magnifying sequencing artifacts, such as mistagging events (Carlsen et al. 2012; Schnell et al. 2015; Esling et al. 2015; Sinha et al. 2017).

Feather mites (Astigmata: Analgoidea, Pterolichoidea) are the most abundant and diverse ectosymbionts of birds (Gaud & Atyeo 1996; Proctor 2003; Doña et al. 2016). They inhabit almost every group (and species) of birds, where they likely act as mutualists or commensalists cleaning bird feathers from fungi and bacteria (Blanco et al. 2001, Proctor 2003). Nevertheless, as for many other symbiont species, several aspects of their biology, ecology and evolution are still unknown and/or controversial (Proctor 2003). This is mainly due to the fact that feather mite species identification is a difficult task; and accurate identification requires advanced taxonomic skills, precluding large-scale studies. DNA barcoding and minibarcoding of single specimens using a region of the COI gene have proved successful for species and specimen delimitation (Doña et al. 2015a) and is becoming standard in species description thus increasing the coverage of DNA barcode libraries (e.g. Dabert et al. 2008; Mironov et al. 2012; Mironov et al. 2015). In addition, DNA metabarcoding using the now-obsolete 454 sequencing technology allowed for the quantification of mites from some species on bulk samples (Diaz-Real et al. 2015). Nonetheless, some technical aspects require further study, and it is needed an adjusted DNA metabarcoding pipeline suitable for large-scale studies using the latest Illumina sequencing technologies.

In this study, we present a series of experiments that show the suitability of the proposed wet-lab and bioinformatic methods for the correct bulk taxonomic identification of feather mite species. Our first experiment investigates the detectability of single individual

mites in mixes of several specimens, as well as the success of the DNA extraction method. Our second experiment analyses the suitability of this method to estimate the relative abundance of mite species in complex samples with mite species having a different number of mismatches in the primer annealing regions. Lastly, we tested the performance of the DNA metabarcoding pipeline proposed here to analyse a sample of 6,501 feather mites from 380 individual birds.

Materials and methods

Sample collection and experimental design

We sorted and identified a total of 5,360 mites. These mites were identified based on morphological characters according to world revisions of the genera *Proctophyllodes* (Atyeo & Braasch 1966) and *Trouessartia* (Santana 1976), and other corresponding taxonomic publications. These mites were included in mixes of known composition in order to construct 94 different mock communities (see below). Each mock community was placed in one well of a 96-well plate and filled with 96% ethanol, leaving two empty wells for a DNA negative extraction control and a PCR negative control. These samples were used for two different experiments:

Experiment 1 (Detecting a single individual in a complex sample): For this experiment, we pooled one individual from seven different species in each of 20 wells of a 96-well PCR plate (i.e. seven individual mites in each of 20 replicates of the experiment). In brief, we performed a DNA extraction, two PCRs (see below) in all of them (i.e. two PCR replicates), and then sequenced both replicates. Then, we looked at the sequences retrieved from our DNA metabarcoding pipeline to evaluate the success in detecting each individual mite specimen.

Experiment 2 (Assessing species abundance in two by two comparisons and assessing replicates differences): For this experiment, we pooled known proportions of different feather mite species into different wells (see details in Table 1).

Table 1. *Number of individuals per species in each well used in all of the two by two comparisons. The species under comparison were: Monojoubertia microphylla vs Proctophyllodes doleophyes, Pterodectes rutilus vs Proctophyllodes stylifer, Sturnotrogus troncatus vs Scutulanyssus hirundicola, Proctophyllodes doleophyes vs Trouessartia kratochvilli, Monojoubertia microphylla vs Scutulanyssus hirundicola, and Proctophyllodes doleophyes vs Dolichodectes hispanicus.*

Species 1	Species 2
1	10
2	10
4	10
6	10
9	10
12	10
17	10
25	10
35	10
46	10
58	10
100	10

In brief, each well contained individuals from two different species. Twelve different proportions were analysed for each pair of species, with two PCR and sequencing replicates each. Altogether, we used eight different species, arranged in six different two by two combinations. We then compared the proportion of individuals in a sample to that of the sequences retrieved from the DNA metabarcoding pipeline. The number of primer mismatches was also taken into account to investigate if this affected the accuracy of the abundance estimates (see Table 2). This was done using previously published mitogenomes or sequences from the species under study (Doña et al. 2015a; Doña et al. 2017).

Table 2. Number of primer mismatches for each feather mite species

Mite species	Forward mismatches	Reverse mismatches	Total mismatches
Scutulanyssus hirundicola	3	0	3
Trouessartia kratochvilli	2	1	3
Sturnotrogus troncatus	1	2	3
Sturnotrogus sp.	1	2	3
Proctophyllodes doleophyes	2	0	2
Monojoubertia microphylla	3	1	4
Proctophyllodes stylifer	3	2	5
Pterodectes rutilus	4	3	7
Pteronyssoides striatus	1	0	1
Dolichodectes hispanicus	3	4	7

Field test: Additionally, a total of 6,501 unsorted, unidentified feather mites were sampled from 380 birds captured across Spain during 2014 (see Table S1, Supporting Material). All of the feather mites collected from each individual bird (i.e. an infrapopulation) weretaken from the wing feathers using a cotton swab soaked with ethanol and preserved a –20 °C in tubes with 96% ethanol. Then, all the mites from the 380 infrapopulations were individually counted under the stereomicroscope and placed into one well of a total of four 96-well PCR plates and filled with 96% ethanol (i.e. each well contained all the mites from one of the 380 individual birds). One well of each plate was left empty as a DNA extraction and PCR negative controls. These samples were used to evaluate the DNA metabarcoding pipeline here proposed to retrieve feather mite-host associations and compare these findings to previously known associations based mainly on morphological identifications (Doña et al. 2016).

DNA extraction

Genomic DNA was extracted from each pool of individuals using the HotSHOT method (Truett et al. 2000): the ethanol was evaporated, a 1-M NaOH solution was added to the dried wells, incubated at 95 °C and neutralised with equivalent amounts of Tris-Cl. The final extraction volume was 30 μL. A negative control that contained no sample was included in every isolation round to check for contamination during the experiments. These controls were

further treated as if they were regular samples. After extraction, the remaining exoskeletons were separated from the buffer and stored in 80% ethanol.

DNA amplification, library construction, and sequencing

All DNA amplicon libraries were prepared by amplifying a region of the mitochondrial COI gene which has been previously tested as suitable for specimen identification and species delimitation while showing within-species polymorphism in feather mites (Doña et al. 2015a, Doña et al. 2015b, Mironov et al. 2015), and by adding the Illumina-specific sequencing primers, indices, and adaptors. This was done following the recommended protocol by Illumina for bacterial 16S metabarcoding, with some modifications. Similar protocols have been used by other authors (e.g. Lange et al. 2014, Vierna et al. 2017). Also, we followed the wet-lab recommendations in Schnell et al. (2015) to minimize cross-contamination events. Specifically, we always used filter tips, the plates were exclusively opened under laminar flow hoods, which were preiodically wiped down with a 0.5% bleach solution, and only one plate was processed per day. The DNA metabarcoding libraries were constructed in a two-step PCR:

PCR1 was carried out using 2.5 μL of DNA as template in a final volume of 25 μL containing 6.50 μL of Supreme NZYTaq Green PCR Master Mix (NZYTech), 0.5 μM of each primer, and PCR-grade water up to 25 μL. The thermal cycling conditions were as follows: an initial denaturation step at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s; annealing at 55 ℃; extension at 72 ℃ for 45 s; and a final extension step at 72 ℃ for 10 min. The primers were the bcd_F05 and bcd_R04 (Dabert et al. 2008) with a 5' overhang that contained the Illumina sequencing primer sequences. A negative control was included in every PCR round to check for cross-contamination during the PCR.

The products of PCR1 were purified by Solid-Phase Reversible Immobilization (SPRI) (Hawkins et al. 1994), using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). In order to eliminate the primer dimers generated during PCR, we used a final bead concentration of 0.5 X, thus size-selecting the high molecular weight amplicons over primer dimers. The purified products were loaded in a 1% agarose gel stained with GreenSafe (NZYTech) and visualized under UV light.

PCR2 was carried out using 2.5 μL of the purified product from PCR1, and the exact same conditions as for PCR1, but using 60 ℃ as the optimal annealing temperature, and only 5 cycles. The primers used for this PCR consisted of a 3' region that anneals to the 5' end of the PCR1 products, and a 5' region that incorporated the adaptors and indices. A total number of 16 forward primers and 24 reverse primers were used for a total of 384 different index combinations. All indices used diferred by at least two bases. The products obtained were purified following the SPRI method as indicated above. Likewise, the purified products were loaded in a 1% agarose gel stained with GreenSafe (NZYTech) and visualized under UV light.

The 96 libraries and their corresponding 96 replicates from Experiment 1 and Experiment 2 (see Experimental design section) were pooled together and run in one MiSeq 300PE run (MiSeq Reagent Kit v3) (MiSeq Run 1 hereafter). Likewise, all 384 libraries from the Field Test experiment were pooled together and run in one MiSeq PE300 run (MiSeq Reagent Kit v3) (MiSeq Run 2 hereafter).

Bioinformatic analyses

The forward (R1) and reverse (R2) fastq reads of each MiSeq run were quality-checked with *FastQC* (Andrew 2010). Then, they were imported into *Geneious 8.1.7* (Kearse et al. 2012) for visual inspection and quality-trimming. We trimmed a region of variable length at the 3' end of each file, according to the average Phred score (minimum quality score of 20) of each MiSeq run. Specifically, for MiSeq Run 1 we trimmed 36 bp and 120 bp from R1 and R2 reads, respectively. Likewise, for MiSeq Run 2 we trimmed 96 bp and 150 bp from R1 and R2 reads, respectively. That way, the length of the reads was identical for all samples in a given MiSeq run. The R1 and R2 files were then exported in FASTA format.

A Python script (MMIS, Supporting Material) was written to automatise the next steps of the bioinformatic pipeline. The R1 and R2 files were concatenated using the *fuse.sh* script available from the *BBmap* package *version 37.00* (Bushnell 2014). Only concatenated sequences with the maximum possible length were kept.

The *split_libraries.py* script included in the pipeline of *QIIME version 1.9.0* (Caporaso et al. 2010) was used to label sequences with the sample identifier and merge them into a unique file per MiSeq run. Then, the *de novo* clustering method and the *UCLUST* algorithm

(Edgar 2010) were used to pick the Operational Taxonomic Units (OTUs) with a 100% of similarity threshold.

A filter to eliminate or minimise mistagging events was implemented. Also referred to as "tag jumps" (Schnell et al. 2015) or "index switching" (Sinha et al. 2017), mistagging is a recently described sequencing artifact that results in the misassignment of reads (generally from 1 to 10 percent) to the wrong sample (Esling et al. 2015; Sinha et al. 2017; Owens et al. 2017). Our filter is based on the rationale that OTUs with a high number of sequences in a sample would "donate" reads, at a low rate, to other samples (Esling et al. 2015). For a given OTU, our filter identifies the sample where that OTU is most abundant in terms of number of reads across all of the samples in a pool. This OTU is treated as the "donor", meaning that it would be the source of the read transfer to other samples. OTUs with a number of sequences below a threshold of 10% of the "donor" OTU or less than 100 reads were filtered out. This conservative threshold was empirically set after observing that a threshold of 6% successfully removed all of the non-expected taxa in our mock communities from Experiment 1 and 2 (where we knew the species included in each well).

After the mistagging filter, the most abundant sequence of each OTU was selected as the representative sequence of that OTU. The *assign_taxonomy.py* script of *QIIME* was used for taxonomic assignment of each representative sequence. Assignment was done with the RDP classifier (Wang et al. 2007) and a minimum confidence score of 96.6% against a reference database (Doña et al. 2015). The reference databases (Appendices 1, 2, Supporting Material) contained one sequence from each of the feather mite species considered in Doña et al. (2015). Since the query sequences spanned the 5' and 3' ends of the reference sequences, but not their central region, the central region of the reference sequences was previously deleted to perfectly match the query sequences. According to the average quality of each MiSeq run, the region deleted from the central part of the reference sequences was slightly different when analysing the results of each MiSeq run (the length of the reference sequences was 298 bp for MiSeq Run 1 and 389 bp for MiSeq Run 2). Then, an in-house *C++* program was used to check if assigned sequences contained STOP codons. And, those sequences with STOP codons were excluded from downstream analyses.

Statistical analyses

All analyses were done in *R* (*v 3.2.3*) environment (R Development Core Team 2015). For those analyses in which the response variable was a rate, we used beta regression tests which is more suitable for modelling non-normal proportional data restricted between 0 and 1 (Ferrari & Cribari-Neto 2004; Bolker 2008). For this, we used the function *betareg* from the *betareg* package (Zeileis et al. 2012). In the model studying the relationship between the total number of mismatches within the primer annealing regions of each mite species and the mean success of detecting it (Experiment 1), the total number of mismatches was the dependent variable and the mean success the independent one. We used the "logit" as the link function. For the analysis of the relationship between the proportion of mites in a sample and the proportion of sequences retrieved (Experiment 2), the proportion of sequences retrieved was the dependent variable, the proportion of mites the dependent one, and we accounted for mite species identity including it as a fixed factor. For the analysis of the relationship between the number of mismatches in the forward primer and the correlation coefficients, the correlation coefficients from Experiment 2 models were coded as the dependent variable and the number of mismatches in the forward primer as the independent one. The relationship between the amplification success (as a binary variable) and the number of mites was studied using Generalized Linear Models (GLMs). We used the *glm* function of the *STATS R* package with a binomial distribution of errors (link "logit) (R Development Core Team 2015). Here, the amplification success was the dependent variable and the number of mites the independent one. We used the *ICCbare* function of the *ICC* package (Wolak et al. 2012) to calculate the intraclass correlation coefficient, that is, to evaluate how repeatable the results from the two replicates of the second experiment were (see above). Lastly, we used the *cor* function from the *stats* package (R Development Core Team 2015) to study the correlation (method "spearman") between the wells of the two replicates.

Results

Sequence statistics and metrics

For MiSeq Run 1 we obtained a total of 18,855,990 reads (R1 + R2). After the bioinformatic cleanup of the sequences, 7,625,864 filtered reads were left. For MiSeq Run 2 we obtained a total of 6,003,886 reads, of which 2,804,200 remained after applying the bioinformatic filters (Table S1, Supporting Material).

Experiment 1. Detecting a single individual in a complex sample

The success of the method to detect single mite specimens in a sample ranged from 10% in *Proctophyllodes stylifer* to 55% in *Scutulanyssus hirundicola*, with a mean success of 33% across all of the species studied, with an average median number of sequences per sample of around 1,600 (Table 3).

Table 3. Probability of detecting a single mite species in a mixture of several specimens. R1 and R2 values refer to those from the first and second replicate, respectively (See Materials and methods).

Mite species	% success R1; R2	min R1; R2	max R1; R2	median R1; R2	Mean % success
Monojoubertia microphylla	25; 45	343; 147	5,190; 10,798	1,096; 936	35
Proctophyllodes doleophyes	30; 40	698; 232	2,804; 25,228	2,721; 2,701	35
Proctophyllodes stylifer	10; 15	204; 144	2,125; 4,423	1,164.5; 406	12.5
Pteronyssoides striatus	40; 40	494; 1,499	15,074; 15,193	2,297.5; 4,144	40
Scutulanyssus hirundicola	25; 55	891; 451	1,628; 19,321	1,223; 3,195	40
Sturnotrogus sp.	20; 40	184; 115	1,171; 810	561.5; 344	30
Trouessartia kratochvili	35; 50	616; 331	7,070; 5,698	1,118; 1,710	42.5

In addition, we found a negative relationship between the total number of mismatches in the primer annealing regions of each species and the mean success of detecting it (betareg: Pseudo R2= 0.50, Z = -2.44; P = 0.015, Fig 1).
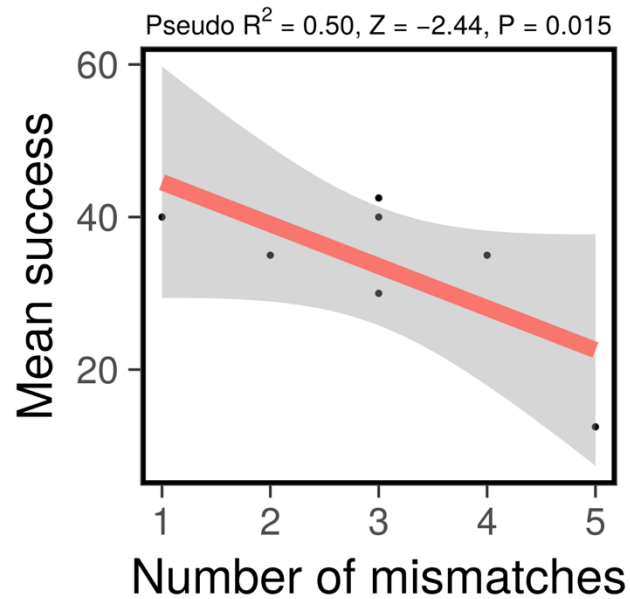
Figure 1. Scatterplot depicting the relationship between the mean success (%) and the total number of primer mismatches. Statistics from beta regression model are given in the top. LM fitted line with 95% confidence interval is shown.

Experiment 2. Assessing species abundance in two by two comparisons and assessing replicates differences

The results of this experiment are summarized in Fig. 2. All comparisons show a statistically significant linear correlation between the proportion of mites in a sample and the proportion of reads retrieved (Fig 2), except for the comparison of *Proctophyllodes styllifer* vs *Pterodectes rutillus*, which was the one with the most overall mismatches in the primer annealing regions (betareg: Pseudo R^2 = 0.27, Z = -0.85; P = 0.40).
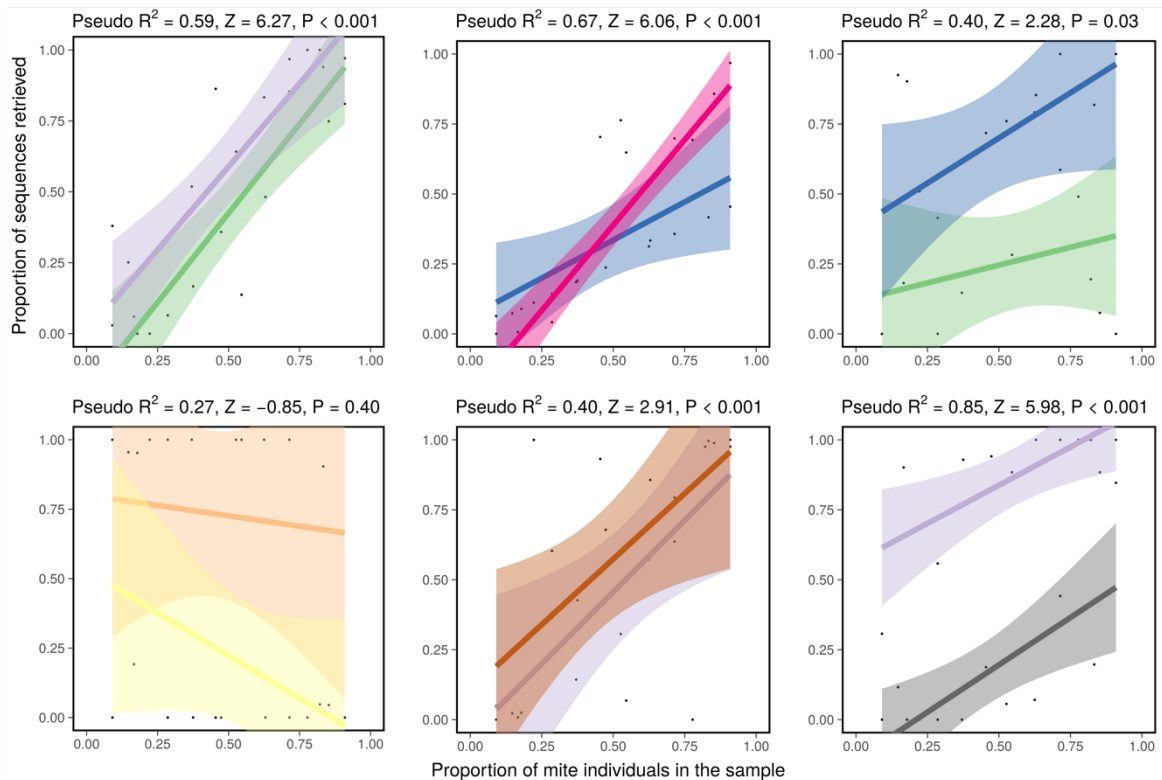
Figure 2. Scatterplot showing the relationship between the number of individuals in a sample vs the number of reads retrieved in two by two comparisons. Colors depict mite species. Purple: Proctophyllodes doleophyes, Green: Monojoubertia microphylla. Magenta: Sturnotrogus troncatus, Blue: Scutulanyssus hirundicola, orange: Proctophyllodes stylifer, yellow: Pterodectes rutilus, brown: Trouessartia kratochvilli, gray: Dolichodectes hispanicus. Note that same species occur in different panels (as explained in Table 1). Statistics from beta regression models are given in the top. LM fitted lines with confidence intervals are shown.

Excluding this non-significant comparison, the results are highly repeatable between replicates, with interclass correlation values ranging from 0.64 to 0.95 (Table 4). In addition, both replicates were highly correlated; we found a significant correlation (Spearman's ρ = 0.78; P < 0.01) for all the wells used for this experiment and Experiment 1 among the two replicates (Fig S2, Supporting Material).

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Table 4. Repeatability between PCR replicates of the proportions in two by two comparisons. Headers from left to right: A) Monojoubertia microphylla vs Proctophyllodes doleophyes, B) Proctophyllodes stylifer vs Pterodectes rutillus, C) Scutulanyssus hirundicola vs Sturnotrogus troncatus, D) Proctophyllodes doleophyes vs Trouessartia kratochvilli, E) Monojoubertia microphylla vs Scutulanyssus hirundicola, F) Dolichodectes hispanicus vs Proctophyllodes doleophyes.

	A	B	C	D	E	F
ICC	0.81	0.55	0.64	0.69	0.73	0.95
Lower CI	0.66	0.27	0.41	0.48	0.55	0.91
Upper CI	0.95	0.83	0.88	0.90	0.92	0.99

Field test. DNA metabarcoding of 380 field infracommunities

All of the samples were sequenced, even when no band was visible in the gel after library construction (N=318, 84%). The proportion of samples for which reads were retrieved was 34%. The success of the method increased with the number of specimens in the sample (GLM: Z = 3.61, df = 382, P < 0.001), getting as high as 53% for samples with more than 30 feather mites, and as low as 22% for samples containing less than 10 individuals (Table 5).

Table 5. Detection success for the different number of specimens in each well.

	<10 mites	10 - 30 mites	> 30 mites
Number of samples	229	82	65
Positives (with reads)	50	42	35
P(x) success	0.22	0.51	0.54

We successfully identified mites from 15 species, comprising four genera (see Table S1, Supporting Material; Fig 3). All bird-feather mite associations retrieved were among those previously reported in the literature and compiled by Doña et al. (2016).

Some OTUs remained unidentified or identified only at the genus or family level (e.g. Fig 3).
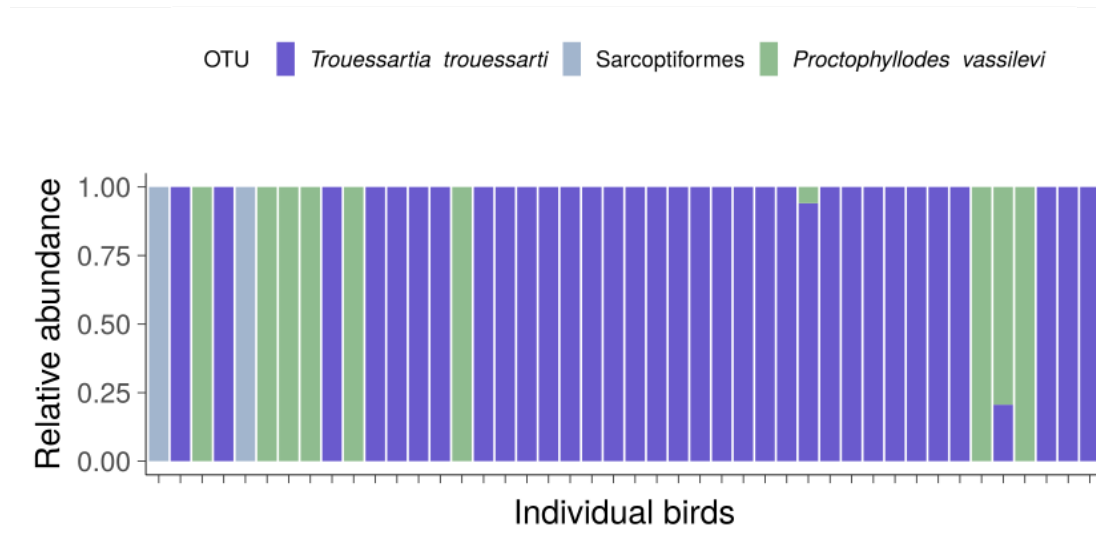
Figure 3. Stacked bar plot showing the mite species retrieved in the mite infrapopulations of the Eurasian Reed Warbler Acrocephalus scirpaceus (Hermann, 1804). Each bar of the plot depicts an infrapopulation.

For these unidentified OTUs, we performed a BLASTn search and we found that: In the case of the samples from the bird genus *Calandrella*, all of the sequences blasted at 96% similarity threshold with *Alaudicola bureschi* (Vassilev, 1958). The expected mite species in this case was *Alaudicola bilobata* (Robin, 1877). However, there are no sequences for *A. bilobata* in the database, so a reliable genetically-based identification to the species level was not possible. In two *Acrocephalus scirpaceus* and one *Acrocephalus schoenobaenus* host individuals we found sequences with 92% similarity with *Trouessartia trouessarti*. Since the taxonomic assignment was performed using a 96.6% similarity threshold, we conclude that those sequences must belong to a new species, or a new host-symbiont association for which no barcode is available yet. A total of 52,717 reads across 55 samples assigned to the genus *Proctophyllodes* could not be identified at the species level (Table S1, Supporting Material). All of them matched with a 100% similarity to species of the *Proctophyllodes pinnatus* species complex, which are not possible to differentiate using the COI region (a case already discussed in Doña et al. 2015a).

Discussion

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Our results show that the proposed DNA metabarcoding pipeline is adequate for the correct identification and for the estimation of the relative abundances of feather mites present in complex samples, especially when there is a moderate number of individuals per sample, and that it is suitable for field samples.

Experiment 1: Detecting a single individual in a complex sample

We evaluated the success of detecting a single feather mite individual in a sample through HTS. The overall success rate was around 30%. This is probably attributable to the DNA extraction method, which yields very little DNA, that was insufficient in many cases. Also, this method does not purify the DNA, as it is coextracted with proteins, lipids and polysaccharides, which may inhibit PCR or decrease the efficiency of the reaction (Schrader et al. 2012).

On the other hand, this method has the advantage of preserving the exoeskeletons for subsequent morphological identification of the specimens (Doña et al. 2015a). This is important for understudied groups such as feather mites, where new species or species with no genetic information are easily found in a field sample. Also, this method is highly practical when dealing with a large number of samples due to its simplicity and low cost in both reagents and time. It is also important to note that this experiment deals with a worst-case-scenario situation. In a real field study, when the whole infrapopulation of feather mites present in a bird specimen is sampled, there should be enough starting DNA, as most birds in some periods of the year (e.g. during pre-breeding stage) can easily have more than 30 feather mites (Diaz-Real et al. 2014).

Experiment 2: Assessing species abundance in two by two comparisons

We compared the proportion of individuals in a sample to that of the reads retrieved, finding a positive, statistically significant relationship between the number of feather mites and the number of sequences for almost all comparisons (Fig. 2). In addition, both PCR and sequencing replicates showed highly consistent results, which was evidenced by the high correlation coefficient (Spearman's ρ =0.78; P < 0.01) found between the wells from the two replicates (Fig. S2, Supporting Material). Therefore, even though it is not possible to infer the

absolute abundance of a certain species in a sample, the method is robust in assessing differences in abundance across different samples. Also, despite the general DNA metabarcoding recommendations of performing several replicates per sample (Ficetola et al. 2015), our results come to show that, at least for this particular group of organisms and using the primers described, performing replicates, though desirable, would not be of paramount importance given the high consistency found here.

The outcome of PCRs performed on diverse mixtures of species is highly dependent on primer specificity and can result in uneven amplification due mainly to mismatches in the primer annealing regions (Sipos et al. 2007). Even though our primers were designed specifically for feather mites (Dabert et al. 2008), and have been used to amplify a wide range of feather mites species (Doña et al. 2015a, b), many species presented several mismatches which affected the amplification success. The total number (forward + reverse) of mismatches ranged from two to seven in the species investigated (Table 2). The best correlation coefficients between the proportion of mite individuals in the sample and the proportion of reads retrieved came from those comparisons where there were fewer overall mismatches. In fact, we found a non-significant negative trend between the number of mismatches in the forward primer annealing region and the correlation coefficients (betareg: Adjusted pseudo R^2 = 0.25; Z = -1.42; P = 0.15, see Fig S1, Supporting Material). Accordingly, future quantitative studies including species which presents a high number of primer mismatches would benefit from using more specific primers, which can be generated using bioinfomatic tools, such as *eco-Primers* (Riatz et al. 2011) or *PrimerMiner* (Elbrecht & Leese 2017) over the growing library of feather mites' COI barcode available at genetic repositories.

Field Test:

The success rate of the method was moderate when the infrapopulations analysed were comprised by more than 30 mites (i.e. 53% of these samples were correctly sequenced, Table 5). However, the overall success of the method was lower. This could be due to the DNA extraction method, as described above, and also to the low feather mite content of many of the samples. This low abundance of mites could be explained by the fact that mites were sampled throughout the year, that is, including the bird's post-breeding stage when the

mite infrapopulation size is much lower due to a massive vertical transmission to chicks (Doña et al. 2017). In view of this, whenever possible, we recommend merging the mites from both wings (and even the tail if the study allows it) into the same sample and sampling mite infrapopulations at those periods of higher abundance of mites (i.e. during the bird's pre-breeding stage and autumn, Pap et al. 2010; Diaz-Real et al. 2014; Doña et al. 2017).

In addition, taking into account the low DNA yield of the extraction method, we recommend dividing each mite sample into two different subsamples whenever possible. This way, it would be possible to use more aggressive DNA extraction methods for one of the subsamples (e.g. mechanically grinding the samples and using a silica column-based DNA extraction method) and thus getting a higher DNA amount of better quality, while preserving the exoskeletons for morphology-based taxonomic work. Here, it is important to stress that preserving morphology is very much needed, considering that most bird species still have to be studied for feather mites and that most feather mite species have probably yet to be discovered (Doña et al. 2016).

Chapter 4.

Vertical transmission in feather mites: insights on its adaptive value

Abstract

The consequences of symbiont transmission strategies are better understood than their adaptive causes. Feather mites are permanent ectosymbionts of birds assumed to be transmitted mainly vertically from parents to offspring. The transmission of *Proctophyllodes doleophyes* Gaud (Astigmata, Proctophyllodidae) was studied in two European populations of pied flycatchers, *Ficedula hypoleuca* Pallas (Passeriformes, Muscicapidae). The vertical transmission of this mite species is demonstrated here with an acaricide experiment. This study also compared (for two distant populations during 4 years) patterns in reductions in mite intensity in adult birds, from egg incubation to chick-rearing periods, with the predictions of three hypotheses on how host survival prospects and mite intraspecific competition might drive feather mites' transmission strategy. The results are in agreement with previous studies and show that feather mites transmit massively from parents to chicks. The magnitude of the transmission was closer to that predicted by the hypothesis based on intraspecific competition, while a bet-hedging strategy is also partially supported.

Introduction

Individual hosts are ephemeral islands for symbionts, making transmission essential for symbiont biology and shaping the symbiont's ecology and evolution (Poulin, 2011; Clayton et al., 2016). Mode of transmission (e.g. horizontal versus vertical, or phoresis versus autonomous movement) is one of the best known life-history traits for many symbiont species, and the ecological (e.g. disease spread) and evolutionary (e.g. parasite virulence) consequences of transmission strategies are well studied (Clayton & Tompkins, 1994; Poulin, 2011; Schmid-Hempel, 2011; Clayton et al., 2016). However, the adaptive reasons for which symbiont species evolve particular transmission strategies remain elusive, and thus ultimate drivers of the diversity of transmission modes are poorly understood.

Permanent symbionts such as feather mites or feather lice living on bird feathers are suitable models to study transmission under natural conditions because they can be studied non-invasively (e.g. Harbison et al., 2008). Moreover, they may be vertically transmitted from parents to offspring in the nest and horizontally in social species, and they can be seen with the naked eye or slight magnification in the field, thus simplifying their study (Clayton et al., 2016). While feather lice are bird parasites and feather mites are most likely commensals or even mutualists of birds (Blanco et al., 2001; Galván et al., 2012), some aspects of their ecology are similar enough to gain some insights from their mutual comparison (Jovani, 2003). For instance, studies on feather lice suggest that species' locomotory capabilities as well as inter- and intraspecific competition are the main drivers of transmission mode (Harbison et al., 2008; Bartlow et al., 2016): species less able to walk when off the host and competitively inferior species (which may obtain benefits from leaving a crowded host in search of a less crowded one) are more likely to disperse via vertical and phoretic transmission (Bartlow et al., 2016). However, additional factors are probably at play in the evolution of transmission strategies. For instance, host survival and symbiont intraspecific competition have been suggested to pose a trade-off in vertical transmission from parents to offspring (Darolova et al., 2001; Brooke, 2010): although nestlings are hosts with relatively low survival prospects compared with adult birds, remaining in the adult host implies higher intraspecific and interspecific competition (Harbison et al., 2008; Brooke, 2010).

Feather mites (Acari: Astigmata: Analgoidea and Pterolichoidea) are among the most abundant ectosymbionts of birds (Proctor, 2003; Doña et al., 2016). Current evidence indicates that they transmit from parents to offspring at the nest through body – body contact during chick rearing (Mironov & Maly- shev, 2002) or among birds in close contact outside the nest (Blanco et al., 1997), and only anecdotally by phoresis (Jovani et al., 2001; Proctor, 2003). Indeed, they cannot survive away from hosts (Dubinin, 1951; Proctor, 2003) and, as a consequence, have evolved adaptations to prevent them from falling off, such as flattened bodies, broad clasping 'feet' (ambulacra) and hooking spines on the body and legs (Mironov, 1999), and behavioural adaptations such as avoiding feathers that are about to be moulted (Jovani & Serrano, 2001).

After previous anecdotal data by Dubinin (1951), the only studies that directly addressed feather mite transmission were those by Mironov (2000) and Mironov and Malyshev (2002), who studied three feather mite species of the common chaffinch *Fringilla coelebs* Linnaeus (Passeriformes: Fringillidae). These studies assumed that all feather mites found on nestlings came from their parents (which is also supported by our experiment; see later) and, thus, that the reduction in feather mite load on breeding adults during the period from egg incubation to chick rearing is caused by this transmission. From the studies of Dubinin (1951); Mironov (2000) and Mironov and Malyshev (2002) (and considering the results here presented), one can extract three important patterns in the transmission of feather mites. First, about three-quarters of the mites on the female parent transmit to her offspring. Second, adults and tritonymphs (the last juvenile stage before becoming adults) are the main mite-transmitting stages. Third, chicks are progressively occupied by mites during their stay on the nest (i.e. when their flight feathers grow), and most chicks had feather mites before leaving the nest.

Such transmission may seem maladaptive because most of the chicks from a nest will never survive (see later) and, consequently, transmission from parents to offspring seems a likely dead end for feather mites compared with those remaining in the adult host. Why, then, do feather mites have this mass transmission to nestlings?

In this study, we investigated empirically the vertical transmission of feather mites in the European pied flycatchers *Ficedula hypoleuca* Pallas (Passeriformes, Muscicapidae). In addition, we tested three hypotheses on the adaptive value of feather mite vertical

transmission by integrating information on adult and chick host survival, the proportion of mites transmitting from parents to nestlings and the distribution of mites among nestlings. From a long-term study (see Camacho et al., 2015 for an overview) in a Spanish population (22 breeding seasons, 4,673 adults and 9,901 chicks ringed) we calculated a 52% probability that breeding adults would reproduce again in the study population (51% for males, 52% for females; J. Potti, pers. comm.). However, nestlings ringed at 13 days old (they leave the nest at age 14 – 20 days; Potti & Montalvo, 1993) have only about a 13% probability of becoming breeders in the studied population in future years (the life expectancy of flycatchers is c. 7 years; Potti, 2000). Even after accounting for a higher emigration among young birds, this shows that passing from an adult to a nestling bird carries a strong risk for feather mites.

From a mite's point of view, the probability of leaving the adult bird host would be:

$$r = 100 \times (1 - (\frac{adult\ survival}{adult\ survival + chick\ survival}))$$. This hypothesis (H1) thus proposes that the decision of feather mites to transmit to a chick or remain on the parent bird is probabilistic (rather than binary) in nature. We propose that natural selection has shaped the sensitivity of mites to particular cues (such as food availability or feather structure) that indicate adult-versus-chick survival prospects. Overall, if feather mites only take this into account for transmission, and given the adult/chick survival expectancies of flycatchers used in this study (see earlier), H1 would predict (assuming that all feather mites behave similarly in this regard) that the reduction in feather mite intensity in parent birds (r) would be 20% from egg incubation to the rearing of fledglings.

This hypothesis, however, does not take into account intraspecific competition in feather mites. For instance, inter- and intraspecific competition have been found to be highly relevant for the transmission of feather lice (Harbison et al., 2008). In addition, current evidence suggests that competition is one factor explaining the habitat partitioning of feather mites (Fernández-González et al., 2015). In fact, feather mites are known to maximise their spread among feathers (i.e. among those they are specialised to live on) and to segregate between the two wings of the bird host; i.e. rather than concentrating in one wing they spread as much as possible, and this is even true for birds having only a few mites (Jovani & Serrano, 2004; Fernández-González et al., 2015). So, in mite infrapopulations (i.e. all the mites living on a given bird), intraspecific competition could favour occupying a lower-quality (i.e. in terms

of survival) habitat if mite density were lower there. Therefore, in a system where the habitat patches (the hosts) are ephemeral and where individuals are so sensitive to habitat loss (if the host dies, the mites will probably die) but competition is present, we hypothesise that the transmission strategy of feather mites may have evolved under a trade-off between high host survival (prioritising less ephemeral hosts: parent birds) and low intraspecific competition (prioritising less crowded hosts: fledglings). Thus, our H2 hypothesis predicts that mites will distribute among all available chicks, but that their distribution will be weighted by the relative survival expectancies of mites on the different hosts (adults versus chicks). In other words, mites would colonise all chicks to take advantage of the relatively lower competition for space (and perhaps for food) on the 'empty' feathers of the chicks, but they would prefer to stay on the parent bird because of higher host survival. So, the number of mites transmitting would depend on a balance between host survival and the number of available hosts. In this scenario, we estimated *r* as for H1, but now, in the denominator, chick survival was multiplied by the number of chicks in the nest (e.g. for a nest with five nestlings, H2 predicts a 60% reduction of feather mite intensity in the parent bird):

$$r = 100 \times (1 - (\frac{adult\ survival}{(adult\ survival + (chick\ survival * number\ of\ chicks))})).$$

Our third hypothesis (H3) does not take into account host survival, but only intraspecific competition, as the main driver of feather mite vertical transmission. Therefore, under our H3 hypothesis, feather mite numbers would decrease in parents according to the number of nestlings in the nest (i.e. by maximising as much as possible the distribution among available hosts) as they do between the two wings of a bird (see earlier). We estimated the expected percentage reduction by dividing 100 by the number of chicks plus one (the parent) as follows: $r = 100 - (\frac{100}{number\ of\ chicks + 1})$. This hypothesis considers that mites leave each parent and pass to the chicks and that, once there, they spread as much as possible. It does not consider the effect of the number of mites present on the other parent, as it is unlikely that both adults would be together in the nest during the mites' transmission.

Materials and methods

Sampling

We investigated the feather mite from European pied flycatchers in two populations from central Spain (see earlier) and the Netherlands (De Hoge Veluwe, 52°2'N, 5°51'W) during 4 years. In both study localities, birds were ringed with metal and coloured plastic bands. Feather mites were counted from primary, secondary and tertiary feathers of the right wing with the naked eye or with the help of a 10× magnifying glass hold- ing the wing up against daylight. Even though this method of counting is error-prone (i.e. because of daily movement of mites or cast skins; Proctor & Owens, 2000), it generally gives good estimates of the number of mites in a given bird (Behnke et al., 1999; Jovani & Serrano, 2004). Moreover, the same observer counted mites within each study unit (e.g. for different chicks from a nest, or for the two measurements of feather mite numbers for a given parent bird), thus avoiding problems arising from inter-observer differences. *Proctophyllodes doleophyes* Gaud (Astigmata, Proctophyllodidae) is the most frequently recorded plumicolous feather mite species of pied flycatchers (Doña et al., 2016). Moreover, this was the species identified in previous studies of Spanish European pied flycatchers, first by Sergey Mironov (Zoological Institute, Russian Academy of Sciences) and afterwards with molecular barcoding (Doña et al., 2015a, 2015b).

A total of 243 individual adult flycatchers were examined for feather mites. In the Netherlands, during the 2009 and 2010 breeding seasons, 55 adult birds were inspected for feather mites twice: both before and after the hatching of their chicks (paired data). Adult male flycatchers (n = 13) were captured inside empty nestboxes in May using clap-traps when the males were still unmated or when their primary female was incubating and they were trying to attract a secondary female to another nestbox. Adult females (n = 42) were captured by hand in the nestboxes during their second week of incubation. Then, adult males and females were recaptured when chicks were 7 – 8 days old. In Spain, during 2000 and 2001, a total of 188 adult birds were sampled either during incubation or while caring for naked chicks (3 days old, i.e. before expected feather mite transmission; Mironov & Malyshev, 2002). One hundred and one of these birds were recaptured when chicks were

12 – 13 days old, thus providing paired data. To reduce the proportion of estimation errors as a result of the small values, the mean and SD calculations of the reductions were done for birds with more than 25 mites during the pre-hatching period, resulting in birds being excluded. In addition, we studied feather mite loads for all nestlings in 24 nests (92 nestlings aged 13 days) from the Spanish population (a subsample of the control nests from the acaricide experiment; see the following section).

Acaricide experimental design

In 2000 and 2001, in La Hiruela, (41∘04'N, 3∘27'W, Madrid, central Spain), we studied the vertical transmission of feather mites from European pied flycatchers. We selected 45 nests in which at least one of the two parents had feather mites during the egg incubation period. We applied three treatments: (i) in 11 nests, we fumigated parent feathers with a common acaricide (Tabernil, Spain) (tetramethrin 0.175%, piperonyl butoxide 0.910%, in gaseous form); (ii) in 28 nests parents were given a sham treatment with water; (iii) in the remaining six nests parents were not treated with either Tabernil or water. Later, when chicks were 13 days old, we inspected the wings of most adult birds (15 and 46 from the acaricide and control treatments, respectively) and all nestlings from all nests for the presence of feather mites. Given the low sample size and the congruence of the results between the two control treatments (see later), data from these control treatments were pooled.

Results

In the acaricide-treated nests, almost all adults (i.e. 80%) completely lost their feather mites (Figure S1), except for two birds that conserved a single mite. In comparison, only 14% of adults in the control nests lost their feather mites entirely (χ^2 = 23.81, P < 0.01; Figure S1). As expected, we did not find any feather mites in chicks from experimental parents, but we detected feather mites in nestlings from 88% of control nests (number of nests positive for feather mite presence at day 13: acaricide treatment, 0/11; control treatment 1, 25/28; control treatment 2, 5/6; χ 2 = 29.20, P < 0.01). Thus, this experiment supports the hypothesis that feather mites found on nestlings come exclusively from their parents and not

via any other means (e.g. from feather mites attached to hippoboscid flies, or mites remaining on the nest-box from a previous year; Jovani et al., 2001).

The study of the variation of feather mites' intensity in nestlings produced interesting results. First, 82% of the chicks had feather mites (Table 1). Second, nestlings' feather mite loads consistently differed between nestlings from different nests (repeatability analysis: $R = 0.64$, 95% CI: $0.46 - 0.82$; Table 1). Lastly, we found that mites showed moderate aggregated distribution among nestlings within nests (variance-to-mean ratio; min = 0; max = 10.31; median = 1.30; Table 1).

Table 1. Descriptive statistics of the number and aggregation of feather mites in flycatcher nestlings (Spain). Two nests containing only one nestling are not shown because they were not used for the aggregation analyses. Variance-to-mean ratio < 1 indicates that mites are evenly distributed, those ~ 1 that the distribution is random and those > 1 that the mites are aggregated (Shaw et al., 1998; Bjørn et al., 2011).

Nest	Nestlings (sorted by mite abundance)								Variance-to-mean ratio
	1	2	3	4	5	6	7	8	
1	14	10	4						2.71
2	14	10	9	7	5				1.28
3	26	7	5	4					10.32
4	2	1	1	0					0.67
5	14	11	8	8					0.80
6	42	25	24	20	14				4.36
7	4	3	1	1	0				1.50
8	17	12	12	11					0.56
9	5	3	2	1	1	1			1.18
10	12	11	6						1.07
11	13	6	4	2					3.67
12	2	1							0.33
13	2	2	1	1	0				0.58
14	1	1	1						0
15	2	1	0	0	0				1.33
16	1	0	0						1
17	8	5	0	1					3.9
18	5	1	1						2.29
19	12	4	2	1	0	0	0	0	7.21
20	10	9	5	2	1				3.02
21	3	2	1	0					1.11
22	2	0	0	0					2

For those females with feather mites in at least one period, we found a sharp 90% reduction in feather mite numbers during the chick-rearing period in both Spain and the Netherlands (Fig. 1) [the Netherlands: paired t-test, $t = 3.44$, d.f. = 38, $P < 0.01$; Spain: paired (birds captured in two periods), Wilcoxon, V = 2346, $P < 0.01$; unpaired (including birds with a single measure), Wilcoxon, $W = 10\ 660.5$, $P < 0.01$]. Similarly, males showed a reduction in the Netherlands (99% of mean reduction; paired t-test, $t = 2.96$, $P = 0.02$) and in Spain

(paired: 71% of mean reduction; Wilcoxon, V = 423, P < 0.01; unpaired: Wilcoxon, W = 662.5, P < 0.01).

Females had larger and consistent decreases in feather mite intensities [generalised linear model (GLM): Z = 8.57; P < 0.01; Fig. 1]. Mite load reductions in males were more variable than in females, and some males (especially those from the Spanish population; Fig. 1) even increased their feather mite load, leading to a significant sex × country interaction on r (GLM: Z = −12.90; P < 0.01; Figs 1, 2). In addition, male and female parents had similar feather mite loads before hatching of the chicks (Spearman's correlation, ρ = 0.51, P < 0.01; Figure S2) and also while rearing chicks (Spearman's correlation, ρ = 0.41, P = 0.02; Figure S3).
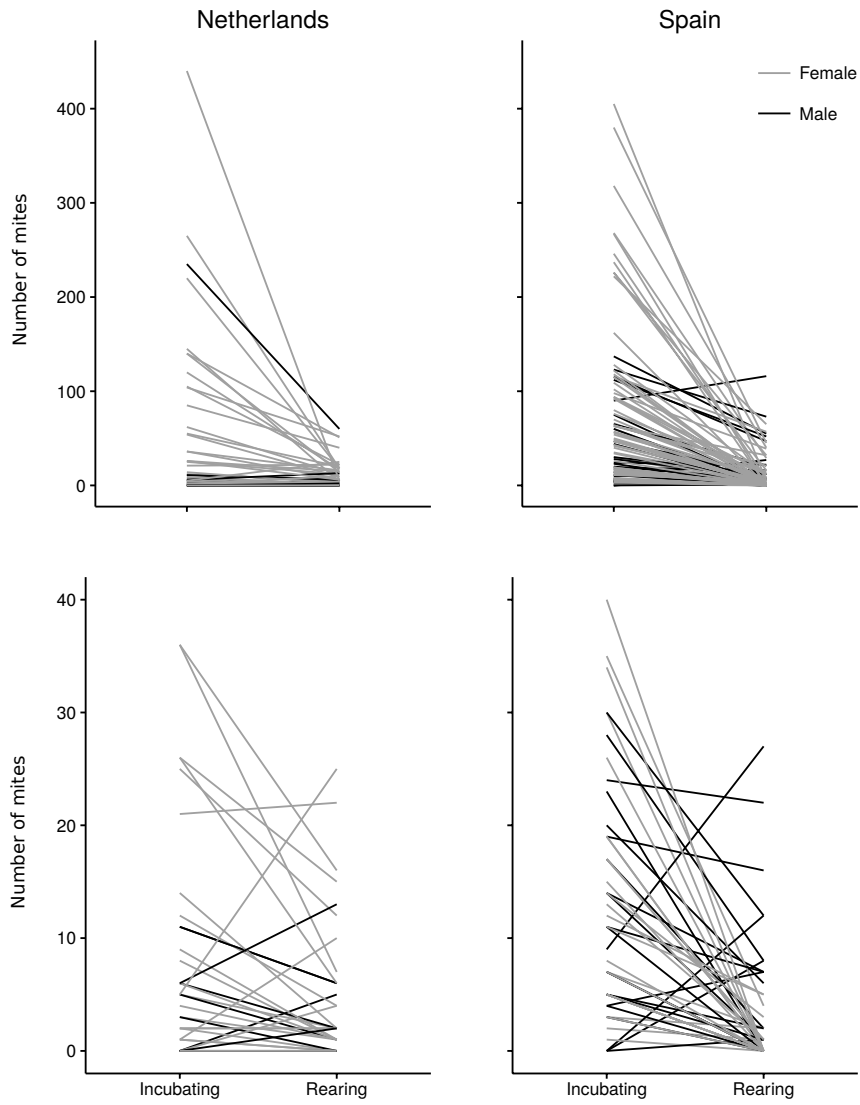
Fig. 1. Feather mite intensity in the same individual adult Ficedula hypoleuca during the incubation and chick-rearing periods. Each line represents an individual bird. Bottom panels are detailed views of the lower part of the top panels (note the change in the y-axis scale) for individuals showing initial mite loads ranging from 0 to 40 mites.

Mite load reductions in both male and female parents in both countries notably exceeded the predictions arising from the relative survival prospects of parents versus nestlings (our H1; Wilcoxon, all $P < 0.05$; Fig. 2) and even exceeded the prediction of our H2 (all $P < 0.05$; Fig. 2). Feather mite reductions were much closer to those predicted by our maximised distribution hypothesis (H3; Wilcoxon, for all except females in Spain, $P > 0.05$; Fig. 2). Nonetheless, even in this case, the reductions exceeded (but by much less) those predicted by H3, thus leading to statistical significance for the group of females in Spain (Wilcoxon, $V = 1899.5$, $P < 0.01$). In fact, H2 and H3 equations probably overestimate mite reductions when both parents have mites. This is because if the other parent is also passing mites to nestlings, mite intraspecific competition would increase in nestlings and thus it may be that fewer mites would pass from parent to nestlings. Therefore, our data show that the transmission is closer to H3 predictions, but even in this case feather mite transmission seems more extreme than is predicted by H3.
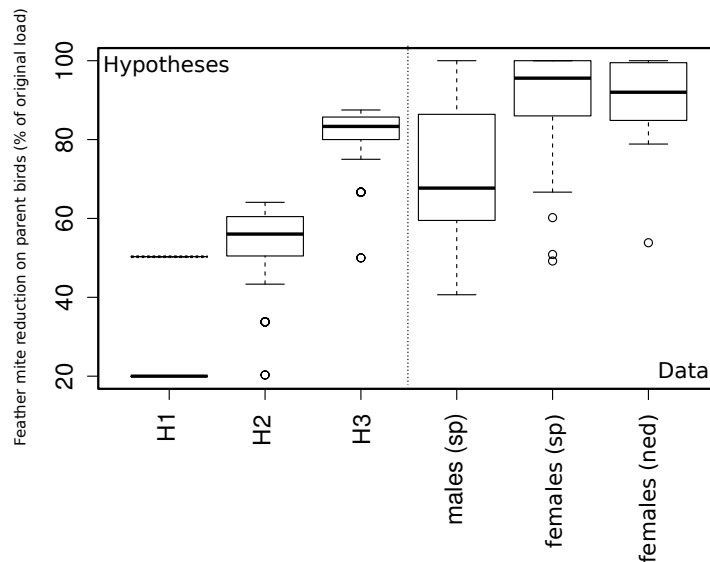
Fig. 2. Boxplots of real and hypothesised feather mite reductions during the breeding season in parent Ficedula hypoleuca. See the main text for explanation of hypotheses H1, H2 and H3. 'sp' refers to Spain and 'ned' to the Netherlands. Spanish males showed a high dispersion in mite reductions. The second predicted value for H1 (dashed line) considers that the probability of survival of nestlings is the same as that of adults (52%). While this may seem unrealistic because nestling survival is expected to be much lower than that of adults (but see main text), it helps to illustrate that feather mite transmission is not explained by the difference in host survival prospects. Note that during the pre-transmission period, all birds with > 25 mites decreased their feather mite loads (see main text). Birds with fewer than 25 mites (during the pre-hatching period) are not shown, for illustrative purposes (n = 5). Similarly, males from the Netherlands population were not included due to the low sample size of males fitting this criterion (n = 2; the median reduction for these males was 99%).

Discussion

These results suggest that the answer to why feather mites transmit massively to nestlings (i.e. a large proportion of the mites leave the parents) may be that the benefits of avoiding interspecific competition exceed the risks of transmitting to a host with lower survival probabilities (the nestling). Thus, the observed transmission patterns of feather mites should be interpreted as if transmitting mites were trying to avoid intraspecific competition by distributing as evenly as possible among available hosts, either adults or nestlings. This scenario is reinforced by the high prevalence (i.e. among nestlings in those nests where parents had mites) and repeatable abundance of feather mites within nests we found. However, the aggregated mite distribution observed within chicks of the same brood suggests that there are other factors (e.g. food resources and/or chick age) promoting among-sibling variation in feather mite loads.

It could be argued that transmission in this system may be the result of despotic competition, where subordinate mites are forced to occupy habitats of lower quality (nestlings instead of adults). However, according to Mironov and Malyshev (2002), tritonymphal and adult mites (the largest stages, and thus likely competitively superior) are the ones involved in transmission. Moreover, we found that the absolute mite reduction in parents was proportional to the number of mites recorded in the same birds before transmission (females, Spain: GLM $Z = 83.06$, $P < 0.01$; Netherlands, GLM $Z = 83.53$, $P < 0.01$; males, Spain, GLM $Z = 21.63$, $P < 0.01$; Netherlands, GLM $Z = 51.82$, $P < 0.01$; Fig. 1). In other words, those birds with more mites also lost more mites. However, the relative reduction (i.e. the percentage of mites lost) was quite constant and unrelated to the number of feather mites before transmission (Spearman correlations, all $P > 0.05$ except for males in Spain). This gives support to the idea that transmitting mite stages are in a similar proportion in different bird parents (Mironov & Malyshev, 2002).

It may seem paradoxical that the most competitive immature stages, because of their size, are the ones leaving the adult bird (Mironov & Malyshev, 2002), i.e. the hosts with higher survival chances and probably the ones providing more food to mites (Haribal et al., 2011). A potential explanation is that tritonymphs and adult mites are those most able to transmit, and thus those whose costs of transmission may be lower (Mironov & Malyshev, 2002). However, a non-exclusive hypothesis may involve inclusive fitness. This is supported by the

way in which population dynamics of feather mites matches the life cycle of birds: feather mites reduce their numbers in birds during bird breeding because of vertical transmission (as shown here) and then increase through the year, reaching a maximum close to the next bird breeding season (Dubinin, 1951; Pap et al., 2010; Haribal et al., 2011, J. Doña et al., pers. comm.). Given the presumed low chance of horizontal transmission of these mites, this may indicate that most of the mites found in a given bird can be close relatives (Doña et al., 2015a) and that infrapopulations may be close to their carrying capacity before bird reproduction (Pap et al., 2010; Haribal et al., 2011). Therefore, negative density-dependence would increase the benefits of transmitting to a new host, not only because it is free of mites (our H3) but because mites are competing for resources with close relatives and thus decrease their own inclusive fitness by remaining in the adult bird. In fact, in a previous study we found that mitochondrial genetic diversity of mite species is highly repeatable between birds of a given species (Doña et al., 2015a). In other words, in a mite species with a high genetic diversity, each bird had several haplotypes (note that an alternative would be that each bird had a single haplotype and that the sum of all birds leads to a high genetic diversity of the mite species). Thus, this may suggest that mites from a given haplotype spread among the different nestlings of a given nest, and that they do not concentrate on a single nestling. This encourages future studies on the trade-off between intraspecific competition and host survival, but which also incorporate inclusive fitness as a likely relevant component for understanding symbiont transmission in genetically isolated systems.

Even if mite lineages spread among hosts (e.g. because of intraspecific competition; H3), this leads to an interesting outcome for mite lineages because the survival probability of a lineage not leaving the adult host would be 52%, but by spreading to the (for instance) five nestlings of the nest, the feather mite lineage increases to 76% the probability that at least one of the hosts will survive until reproduction, and thus until the next transmission opportunity for mites. Thus, it is possible that feather mite lineages increase their survival expectancies by spreading the risk of mortality among the available hosts, i.e. a bet-hedging strategy (Fenton & Hudson, 2002).

Constraints may also be involved in feather mite transmission. In fact, bottleneck genetic signatures have been found for most feather mite species studied to date (Dabert et al., 2015; Doña et al., 2015a). In this study, female pied flycatchers (i.e. the

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incubating/brooding sex and thus the one having more direct contact with nestlings) had higher and consistent decreases in feather mite intensities. In addition, we found that pair mates had similar feather mite loads before the hatching of the chicks, suggesting either mite horizontal transmission between adult birds in the nests or assortative mating according to some individual trait linked to feather mite load (Blanco et al., 2001). Moreover, the few males that increased their mite load may suggest a much higher reproduction of mites on the adult male, which surpassed the number of mites transmitted to nestlings (but little is known about the feather mites' generation times), or transmission of mites from the female (including extra pair mates; see later) and also indirectly through short contacts with nest material to the adult male.

Overall, our findings support the view that variation in the behaviour of breeding birds may constrain the opportunities for feather mite transmission. A previous study in chaffinches found that feather mite loads did not decrease in male birds along the breeding season (Mironov & Malyshev, 2002). Indeed, here we found that some males increased their feather mite load (Fig. 1). These differences could be explained by the differences in mating system as, unlike chaffinches, pied flycatcher males are frequently polygamous and interact with several females (Cramp & Perrins, 1994; Canal et al., 2012). Further work is encouraged here.

Research into adaptive strategies of symbiont transmission is in its initial stages, but recent studies, adopting concepts already tested in dispersal studies of free-living organisms (e.g. by testing condition-dependent dispersal in symbionts; Skelton et al., 2015), are promising. In fact, the results reported here for feather mites could be easily extended to other vertically transmitted symbionts. Experiments modifying the intensity of competition in symbionts would allow the effect of intraspecific competition on the outcome of transmission to be tested directly. The contribution of inclusive fitness to symbiont dispersal strategies also needs more attention. Further research along both research agendas will be relevant to achieve a more complete picture of the ecology and evolution of host– symbiont systems.

Chapter 5.

Feather mites play a role in cleaning host feathers: New insights from DNA metabarcoding and microscopy.

Abstract

Parasites and other symbionts are crucial components of ecosystems, regulating host populations and supporting food webs. However, most symbiont systems, especially those involving commensals and mutualists, are relatively poorly understood. In this study, we have investigated the nature of the symbiotic relationship between birds and their most abundant and diverse ectosymbionts: the vane-dwelling feather mites. For this purpose, we studied the diet of feather mites using two complementary methods. First, we used light microscopy to examine the gut contents of 1,300 individual feather mites representing 100 mite genera (18 families) from 190 bird species belonging to 72 families and 19 orders. Second, we used high-throughput sequencing (HTS) and DNA metabarcoding to determine gut contents from 1,833 individual mites of 18 species inhabiting 18 bird species. Results showed fungi and potentially bacteria as the main food resources for feather mites (apart from potential bird uropygial gland oil). Diatoms and plant matter appeared as rare food resources for feather mites. Importantly, we did not find any evidence of feather mites feeding upon bird resources (e.g., blood, skin) other than potentially uropygial gland oil. In addition, we found a high prevalence of both keratinophilic and pathogenic fungal taxa in the feather mite species examined. Altogether, our results shed light on the long-standing question of the nature of the relationship between birds and their vane-dwelling feather mites, supporting previous evidence for a commensalistic–mutualistic role of feather mites, which are revealed as likely fungivore–microbivore–detritivore symbionts of bird feathers.

Introduction

Symbionts (i.e., parasites, mutualists and commensalists that intimately interact with their hosts; Leung & Poulin, 2008) comprise the most diverse group of organisms on Earth (Dobson et al. 2008; Larsen et al. 2017; Poulin & Morand, 2000, 2004). Symbionts are crucial for ecosystem stability: they regulate host populations and support food webs, where parasites alone are responsible for 75% of the network links (Lafferty, et al. 2006). Thus, the study of host–symbiont ecology is vital to understand many important processes, such as emerging infectious diseases (Hoberg & Brooks, 2015), biological invasions (Traveset & Richardson, 2014), crop pests (Hosokawa et al. 2007) or the effect of climate change upon biodiversity (Carlson et al., 2017). Historically, most efforts have been directed to the study of parasites with direct harmful effects on humans or livestock. Symbiont systems involving commensals and mutualists are relatively poorly studied compared to free-living organisms and host–parasite systems (Jovani et al. 2017).

Host–symbiont interactions rarely involve a simple one-symbiont:one-host interaction. Rather, even without considering the interaction of the host species with other free-living species, any host–symbiont interaction typically involves several other species (Hopkins et al. 2017; Poulin, 2010). In addition, whether a particular symbiont species acts as a parasite, commensal or mutualist can be highly context-dependent (i.e., the mutualism–parasitism continuum framework; for example, Brown et al. Farrell, 2012; Cheney & Côté 2005; Newton et al. 2010; Jovani et al. 2017). Thus, the study of symbionts as a whole, and not separately according to the presumed nature of their relationships with their hosts, is needed (Jovani, 2003; Jovani et al. 2017).

Defensive mutualisms (i.e., those in which symbionts protect their hosts from natural enemies, which have been often perceived as biological curiosities) have been reviewed recently following this approach and placed into this framework (Hopkins et al. 2017). Accordingly, defensive mutualisms, instead of being anecdotal host–

symbiont associations, have been revealed as diverse and common associations in a wide range of plants and animal hosts from nearly all habitats on the planet. Nonetheless, with a few exceptions, most of the diversity of host–symbiont associations remains unexplored or largely unstudied.

A good example of our lack of knowledge of these interactions involves symbiotic relationships between birds and their feather mites (Acariformes: Astigmata: Analgoidea and Pterolichoidea). These mites are the most abundant and diverse ectosymbionts of birds. Almost all bird species harbour species- or genus-specific feather mites (Doña et al. 2016; Gaud & Atyeo, 1996; Proctor, 2003). Feather mites are highly specialized symbionts due to their (i) life cycle (i.e., they are permanent ectosymbionts, Dabert & Mironov, 1999; Proctor, 2003); (ii) high host specificity (Doña et al. 2017); (iii) specific distribution on particular feathers and microsites on feathers (Fernández-González et al. 2015; Jovani & Serrano, 2001, 2004; Stefan et al. 2015); and (iv) mainly vertical mode of transmission (Doña et al. 2017; Jovani et al. 2001; Mironov & Malyshev, 2002). However, as with many other symbionts, they are challenging to study, and this has strongly hampered our comprehension of this system (Doña et al. 2015; Proctor, 2003; Proctor & Owens, 2000).

A long-standing question in understanding the interaction between feather mites and birds is whether these mites feed on bird tissues (e.g., feathers, skin, blood) or upon resources found on the bird's surface (e.g., algae, fungi). If they feed on bird tissues, they are more likely to be classified as parasites (Harper, 1999; Poulin, 1991; Thompson et al. 1997), while if they do not, feather mites would more likely be commensals or even mutualists (Blanco et al. 1997, 2001; Galván et al. 2012). Previous evidence has suggested that feather mites could feed mainly on the uropygial gland oil of birds (Dubinin, 1951; Proctor, 2003; Walter & Proctor, 2013c). However, this oil is a nitrogen-deficient source (Jacob & Ziswiler 1982; Proctor, 2003), and previous evidence has shown that feather mites complement their diet

with fungi, pollen and algal particles (Blanco et al. 2001; Dubinin, 1951; Proctor, 2003; Walter & Proctor, 2013c). Examining thousands of slide-mounted feather mites from 26 mite species, Dubinin (1951) found that almost all mite species had fungal spores in their guts, most from *Cladosporium*, *Alternaria* and rust fungi. Moreover, Blanco et al. (2001) found fungal mycelia and spores in the guts of 53% of *Pterodectes rutilus* (Robin) (Proctophyllodidae) and 38% of *Scutulanyssus nuntiaventris* (Berlese) (Pteronyssidae) mites from two species of swallows (Hirundinidae). Likely because of this potential mixture of feather mite diet, a recent isotopic study (Stefan et al. 2015) of the diet of two feather mite species produced inconclusive results. Interestingly, however, this study showed a strong correlation between the isotopic carbon signatures among mites inhabiting the same individual host, and between the carbon signature (but not the nitrogen signature) of feather mites and the blood of their individual bird host, thus suggesting that diet could be mainly based on shared host-associated resources, arguably preen gland oil (Stefan et al. 2015). Thus, it remains an open question to what extent feather mites feed on uropygial oil or also upon other bird tissues, whether exogenous resources, such as fungi and bacteria, constitute an important food resource for these mites, and which specific taxa are eaten by feather mites.

In this study, we investigated the diet of feather mites using two complementary methods. First, we used light microscopy to examine feather mite gut contents under the microscope from a large sample of feather mites from ~ 200 bird species. Light microscopy allows detection of feather fragments, fungi, plant material and algae that are refractory to the clearing and mounting media (see Materials and methods). In a second approach, for a smaller number of vane-dwelling mite species, we studied gut contents using high-throughput sequencing (HTS) and DNA metabarcoding. This molecular approach complemented the light microscope analysis for certain potential food resources that would not be easily recognized in

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the slide-mounted specimens (e.g., bacteria, soft bird tissues) and also allowed for a detailed analysis of fungi, bacteria and plant taxa in the mites' diet.

Materials and methods

Gut content assessment via light microscopy

For the microscopy analysis, we used previously slide-mounted mites from the Proctor Lab collection of feather mites from around the world. Mites had been cleared in lactic acid and mounted in polyvinyl alcohol medium (#6371A; BioQuip, Rancho Dominguez, CA, USA). This process clears soft tissues but retains refractory material (e.g., chitin, cellulose). Selection of mites to examine was based on taxonomic diversity of mites and host birds, and ecological breadth of hosts (e.g., birds from terrestrial, marine and freshwater habitats, including predators, granivores, nectarivores, etc.). We initially examined several thousand mites using a Leica DMLB compound microscope with DIC lighting. Mites with visible gut contents were photographed at various magnifications (200, 400 and 800x) depending on size of material in the gut. For each host bird species included in the study, our goal was to photograph a minimum of five individual mites from each mite genus present on the bird species. In some cases, if there were fewer than five mites with gut contents available for a mite genus and/or bird species, then all the available mites that contained gut contents were photographed. Under ideal circumstances, we would have focused on mite species rather than genera, but particularly for tropical areas, feather mite alpha-taxonomy is in an early state and many species have yet to be described. Also, for many taxa, only adult males can be readily ascribed to species, and we wished to include nymphal and female mites in our assessment. Mites were identified to genus using Gaud and Atyeo (1996) with additional literature for more recently described genera (e.g., Valim & Hernandes, 2010). In total, 1,300 individual mites representing 100 genera (18 families) from 190 host bird species (72 families; 19 orders) were photographed.

Each morphologically unique type of gut content was given a code, and for every individual mite, all the types of gut content present were recorded, as well as the approximate amount of each type of gut content. Aided by illustrations in Lacey and West (2006) and

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consultation with a mycologist (T. Spribille, University of Alberta), we then classified all unique types of gut contents as fungi, diatoms, plant spores, "unidentifiable" and oily globules (possibly uropygial gland oil or digestive by-products in peritrophic membranes). Unidentifiable objects were mainly extremely small fragments or flecks of material <5 μm long (some of which could have potentially been tiny remnants of feather barbules) (e.g., Figure S10). Oil globules were not included in the analyses, as we consider that our ability to consistently identify this material was much lower than for other types of gut content (see an example of potential oil globs in Figure S11).

Sample collection and sterilization for DNA metabarcoding

For the DNA metabarcoding study, 1,833 individual mites of 18 mite species from 18 passerine bird host species (34 individual birds or infrapopulations) were sampled from birds captured with mist nets in Andalusia (Spain) during the spring of 2015 (see Table S1, for sampling details). An effort was made to collect all mites found on the wing flight feathers from each sampled bird, using a sterile swab impregnated with ethanol. Mites were preserved at -20°C in tubes with 96% ethanol. In those cases in which more than one mite species was found on an individual bird, one different sterile swab was used for collecting each tentative mite species (according to Doña et al. 2016 based on genus-specific location on bird feathers) into different tubes.

Mites were sterilized in AllGenetics & Biology, SL (A Coruña, Spain) with three ethanol washes following Andrews (2013). Each time, tubes containing mites were agitated manually. Then, all ethanol was collected with the pipette using a thin pipette tip, with careful visual checks to avoid removing any mites. Tubes were then refilled with ethanol. Washed mites were then used for further analyses (hereafter mite samples) and the ethanol extracted from the first wash was used as the environmental control sample (hereafter, external sample).

DNA extraction, amplification, library construction and sequencing

DNA isolation, amplification and library preparation were carried out at AllGenetics & Biology, SL (A Coruña, Spain). Genomic DNA was extracted from each mite sample using the HotSHOT method (Truett et al. 2000). Briefly, the ethanol from the last mite wash was

evaporated and a 1-M NaOH solution was added to the dried wells, incubated at 95°C and neutralized with equivalent amounts of Tris–Cl. The final extraction volume was 30 μl. A negative control that contained no sample was included in every extraction round to check for contamination during the experiments. This procedure preservesexoskeletons for morphological identifications (see Doña et al. 2015). However, in contrast to more aggressive isolation methods, DNA from Gram-positive bacteria, undigested diatoms and intact fungal spores may not have been amplified. After DNA extraction, the remaining exoskeletons were separated from the buffer and stored in 80% ethanol. External samples were extracted as follows. The ethanol phase from the first mite wash was pipetted onto a nitrocellulose filter (ca. 9 cm² with a pore size of 22 μm), and then, DNA was isolated using the PowerSoil DNA isolation kit (Mobio) following manufacturer's instructions. The final elution volume was 50 μl.

From each sample, a total of seven libraries were built: five from DNA extracted from mite samples and two from the DNA extracted from the external samples (i.e., see above for sample name definitions). HTS libraries were prepared by amplifying a different molecular marker and by adding the Illumina-specific sequencing primers, indices and adaptors. The regions amplified from mite samples were as follows: the bacterial/archaeal 16S rRNA gene variable region 4 (515F/806R, Caporaso et al. 2012), the ITS 2 region of the fungal rRNA operon (ITS86F/ITS4, De Beeck et al. 2014), the ITS 2 region of plants and algae (S2F/S3R, Chen et al. 2010) and the region of the mitochondrial COI gene of birds. To maximize the potential for retrieving bird DNA, we used internal primers of the mitochondrial COI gene suitable for amplifying degraded DNA (BirdF1/AvMiR1, Kerr et al. 2009). In addition, we amplified the COI gene of feather mites (bcdF05/bcdR04, Dabert et al. 2008) to molecularly confirm the mite species identity (Doña et al. 2015). Only bacterial and fungal regions were amplified from the external samples.

Libraries were built following the recommended protocol by Illumina for bacterial 16S metabarcoding, with some modifications. Similar protocols have been used by other authors (e.g., Lange et al. 2014; Vierna et al. 2017). Briefly, the libraries were constructed in a two-step PCR (hereafter, PCR1 and PCR2): PCR1s were carried out in a final volume of 25 μl, containing 6.50 μl of Supreme NZYTaq Green PCR Master Mix (NZYTech), 0.5 μM of each primer and PCR-grade water up to 25 μl. Thermal cycling conditions included an

initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at various temperatures (bacteria: 50°C; fungi: 52°C; plant: 51°C; bird: 59°C; mite: 55°C), extension at 72°C for 45 s and a final extension step at 72°C for 10 min. PCR1 products were purified by solid-phase reversible immobilization (SPRI) (Hawkins et al. 1994), using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). To eliminate the primer dimers generated during PCR, we used a final bead concentration of 0.5X, thus size selecting the high molecular weight amplicons over primer dimers. The purified products were loaded in a 1% agarose gel stained with GreenSafe (NZYTech) and visualized under UV light.

PCR2 was carried out using 2.5 μl of the amplified DNA from PCR1 as a template and was performed under the same conditions as PCR1, but only running five cycles at 60°C as the optimal annealing temperature.

A total of 31 different index combinations were used, and 40 PCR cycles were performed (Vierna et al. 2017). The resulting products were purified following the SPRI method as indicated above. Likewise, the purified products were loaded in a 1% agarose gel stained with GreenSafe (NZYTech) and visualized under UV light.

All products (a total of 238 libraries) were pooled together in 21 sets of differentially indexed samples. All pools were quantified with Qubit™ fluorometer (Invitrogen). We did not obtain bird DNA in any sample and plant DNA only from two samples (see Results below). Accordingly, all except one plant pool (i.e., the one containing the only two samples successfully amplified, see Results below) were not sequenced as they did not reach the minimum amount of DNA for HTS.

All pools were sequenced by Novogene (Beijing, China) on Illumina HiSeq 4000 using the PE 250 strategy (see Supporting Information for coverage information; Table S2). Quality controls were carried out using company in-house Perl scripts to remove contaminated adaptors and low-quality sequences.

Bioinformatic analysis

Bacterial sequences were postprocessed and classified with *MOTHUR v1.38.1* (Schloss et al. 2009) according to the *MiSeq SOP* (accession date: 30 August 2016, Kozich et al. 2013). In brief, sequences were aligned and classified against the *SILVA (v123)* database (Pruesse et al. 2007). Potential mitochondrial, chloroplastidial and other nontarget sequences were

removed, and the *UCHIME* algorithm was used to identify and remove chimeras (Edgar et al. 2011). Lastly, sequences were clustered into OTUs using the *cluster.split* command. Fungal sequences were processed using the *PIPITS* pipeline (Gweon et al. 2015). Briefly, this procedure extracts the ITS subregion from reads and then assigns them taxonomically with a trained RDP Classifier (Bengtsson-Palme et al. 2013). One mite sample containing <100 reads after preprocessing was not used for further analyses on fungal sequences (see Table S2). Plant raw reads were quality trimmed (sliding window of 30 bp with a minimal average Phred score of 33) using *TRIMMOMATIC 0.36* (Bolger et al. 2014) and then clustered to OTUs at 97% using *CD-HIT version 4.5* (Fu et al. 2012). Representative (centroid) sequences were blasted using *MEGABLAST* against the NCBI "nr" nonredundant nucleotide sequence collection (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/).

Mite identity was molecularly confirmed in all cases using a similar pipeline to that used in Doña et al. (2015). In brief, we used Geneious R10 (http://www.ge neious.com, Kearse et al. 2012) plugin *Sequence classifier*, over a concatenated file containing the forward and reverse reads (quality trimmed as described above for plant libraries and with a minimum length of 200 bp). Then, we used the recommended threshold and a reference DNA barcode library (Doña et al. 2015).

Statistical analysis

Differences in prevalence and morphological diversity of diet resources (the maximum diversity retrieved for each mite sample, that is, each mite infrapopulation; see above) from microscopy assessments were analysed using generalized linear mixed models (GLMM) (*GLMER* function from package *LME4 1.1-12*, Jovani & Tella, 2006; Bates et al. 2015). For assessing differences in prevalence, we ran a binomial GLMM considering prevalence (1: presence, 0: absence) as the response variable, the type of food resource as the predictor variable and the bird infrapopulation nested into bird species plus mite genera as random factors. For assessing differences in morphotype diversity of fungi and diatoms, we ran a Poisson GLMM considering morphotype diversity as the response variable, and the same structure of predictor and random factors. We confirmed assumptions underlying GLMMs by exploring regression residuals for normality against Q-Q plots.

Fungal and bacterial OTUs were imported to *R* and manipulated using *PHYLOSEQ R* package (McMurdie & Holmes, 2013). In particular, we studied the variance in bacterial and fungal assemblage composition among infrapopulations using a permutational multivariate analysis of variance on Bray–Curtis and Jaccard distance matrices (PERMANOVA; *adonis* function from the *VEGAN v2.4.1 R* package, Oksanen et al. 2017). The null hypothesis was that the centroid does not differ between host species and/or mite species (Anderson & Walsh, 2013). This test is highly sensitive to data dispersion (Anderson, 2001), and thus, we tested it with the multivariate homogeneity PERMDISP2 procedure (Anderson, 2006; *betadisper* function from *VEGAN*, Anderson & Walsh, 2013) with 999 permutations. Additionally, following previous approaches to overcome this statistical issue (e.g., Brice et al. 2017), we explored the community clustering with ordination analyses (principal coordinates analyses, PCoA) and stacked bar plots at the infrapopulation level.

Results

Composition and morphological diversity of feather mites' diets assessed by microscopy

From a total of 481 infrapopulations (1,300 individual mites) belonging to 190 bird species and 100 mite genera, fungal material (spores and hyphae) was the most prevalent type of gut content (GLMM: $x^2 = 168.73$, df = 2, $p < .001$; Figure 1) and the most morphologically diverse (GLMM: $x^2 = 442.5$, df = 2, $p < .001$; Figure 1). In addition, diatoms and plant material were also found, but in a much lower frequency and morphotype diversity than fungi (Figure 1). Highly similar results were found when only analysing passerines (Figure S1 and S2), the avian order in which bird species were also studied using DNA metabarcoding (see below). The overall predominance of fungi was widespread across the avian phylogeny (Figure 2) and feather mite taxonomy (Table 1).
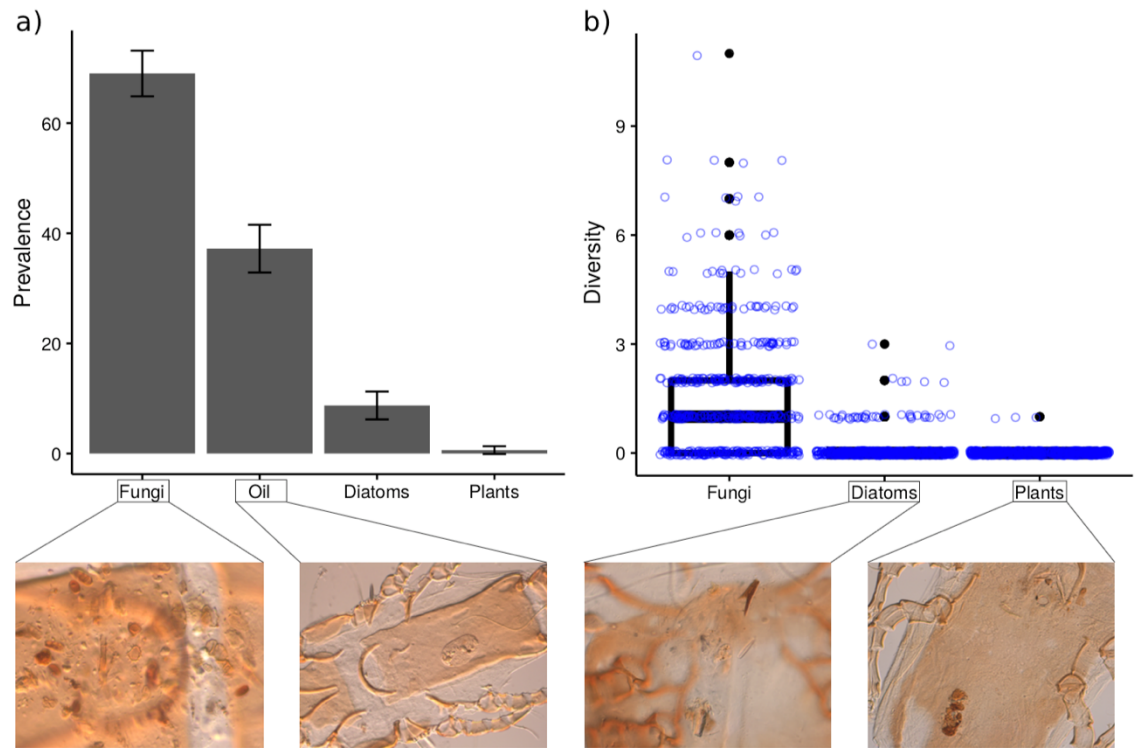
FIGURE 1 Barplot and boxplot depicting the (a) prevalence (N = 481) and (b) morphological diversity (using the maximum diversity retrieved per infrapopulation) of diet items found in the microscopy assessment of feather mite gut contents. Error lines in (a) represent confidence intervals (95%). Blue dots in (b) represent real data points (jittered). Representative pictures of each food resource are placed beneath the plots.

TABLE 1 Prevalence (% of feather mite infrapopulations) of identified food items found in the best-sampled mite families. Phylogenetic information was retrieved from Klimov and O'Connor (2013).

Mite family	N	Fungi	Diatoms	Plants
Proctophyllodidae	162	53	10	1
Alloptidae	22	23	9	0
Trouessartidae	29	48	3	0
Pteronyssidae	51	61	6	0
Xolalgidae	22	64	14	0
Psoroptoididae	23	48	0	0
Analgidae	59	41	2	0
Avenzoariidae	28	32	25	0
Pterolichidae	28	54	7	1
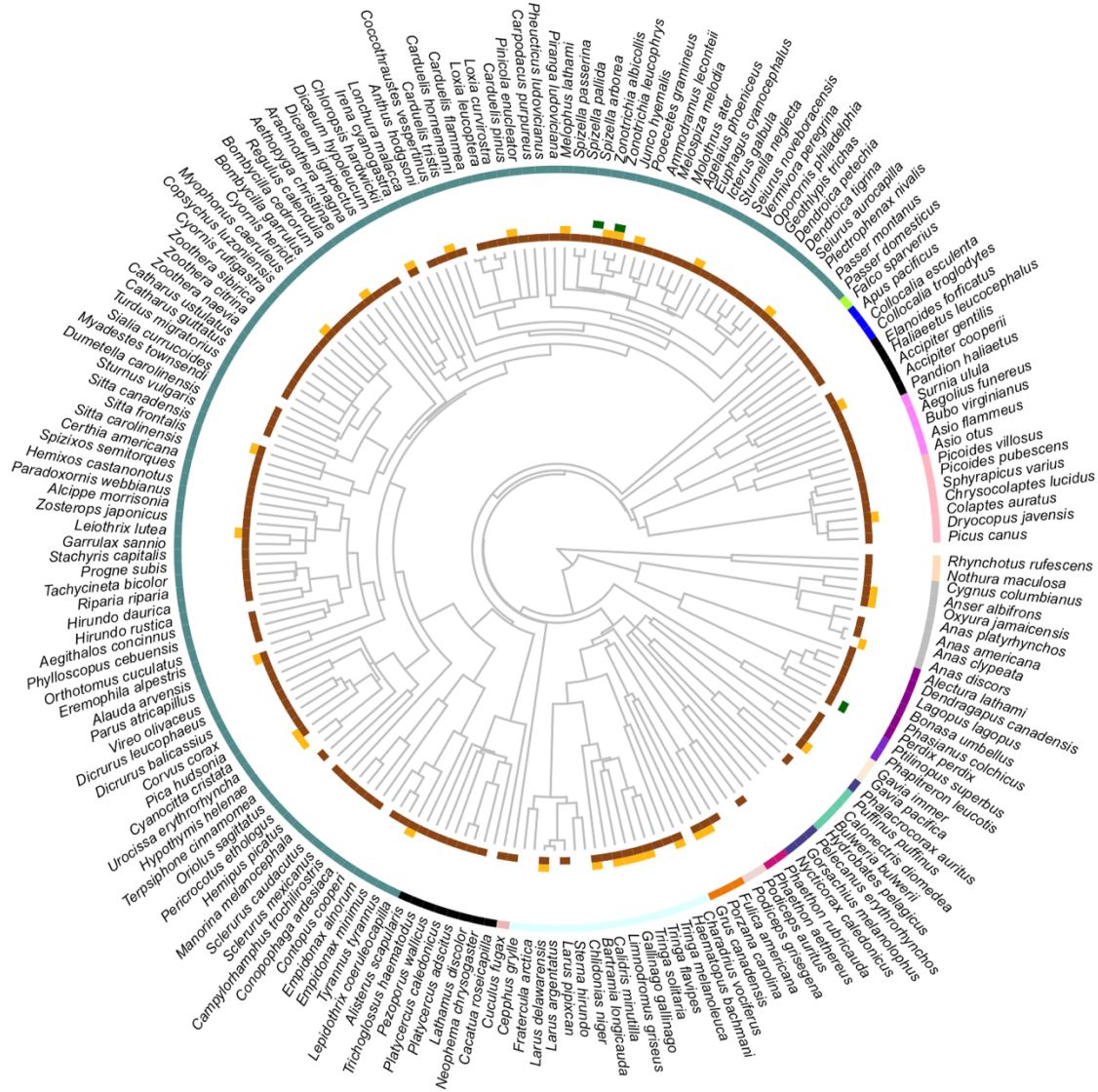
FIGURE 2 50% majority-rule consensus phylogenetic tree depicting the distribution of food resources retrieved by microscopic analysis of feather mite gut contents across the phylogeny of birds. In brief, 1,000 trees were obtained from BirdTree (Jetz et al. 2012, http://birdtree.org) and summarized using SUMTREE v 4.1.0 in DENDROPY V4.1.0 (Sukumaran & Holder, 2010, 2015), following Rubolini et al. (2015). Rings from the centre out, brown: fungi. Mustard: diatoms. Green: plants. Most external ring colours depict bird orders

DNA metabarcoding of feather mites' diets

Metabarcoding results of the mite species from the genera *Proctophyllodes* Robin, 1877, *Trouessartia* Canestrini, 1899, *Dolichodectes* Park & Atyeo, 1971, and *Scutulanysuss* Mironov, 1985 showed highly congruent results with the microscopic analyses in terms of

the prevalence and diversity of food resources, while complementing them with bacterial detection and providing taxonomic detail of the organisms involved. We found bacterial DNA in all samples (Table S2). The bacterial genera identified primarily belonged to the phyla Proteobacteria, Actinobacteria and Bacteroidetes, with Proteobacteria being the most frequently represented (Figure S5). Within these phyla, we retrieved a high diversity of bacterial genera (Figures 3, S7 and S8). Genera commonly found in soil and as environmental "background noise" such as *Sphingomonas*, *Acinetobacter* and *Pseudomonas* were the most prevalent genera (Table 2, Figures 3, S7 and S8) while typically endosymbiotic genera such as *Bartonella*, *Enterococcus* and *Buchnera* were the most abundant when they were present (Table 2, Figures 3, S7 and S8). PERMANOVAs showed statistically significant differences in bacterial composition between mite (53% variance, $F = 1.25$, $p = .006$) and bird species (52% variance, $F = 1.31$, $p = .001$). Nonetheless, we found different levels of dispersion between mite ($F = 7.19$, $p = .001$) and bird species ($F = 9.95$, $p = .001$). In addition, ordinations as well as individual stacked bar plots of bacterial profiles did not show clustering by mite or by bird species in bacterial OTUs or genera (Figures 4 and S7). Additionally, a re-analysis excluding all bacterial OTUs found in the external samples, that is, to exclude potential environmental contamination coming from bacterial OTUs still remaining after mite washes, showed almost identical results: significant differences in bacterial composition between mite species (PERMANOVA, 51% variance, $F = 1.15$, $p = .023$) and bird species (PERMANOVA, 49% variance, $F = 1.20$, $p = .01$). Nonetheless, again, we found different levels of dispersion between mite species ($F = 8.46$, $p = .002$) and between bird species ($F = 11.84$, $p = .001$). In addition, ordination and profile plots did not show clustering by either mite or bird species in bacterial OTUs and genera (Figures S8 and S9).
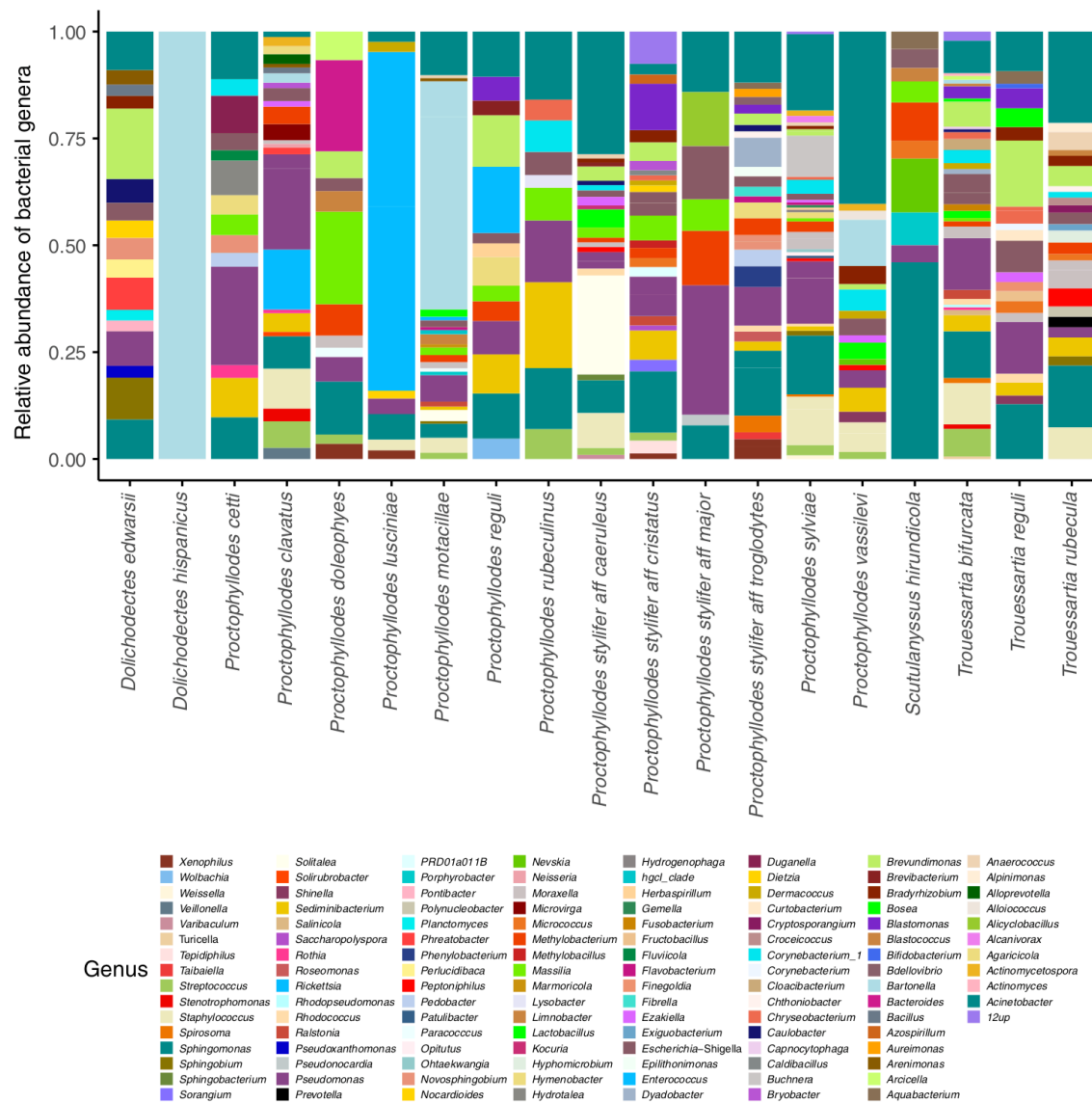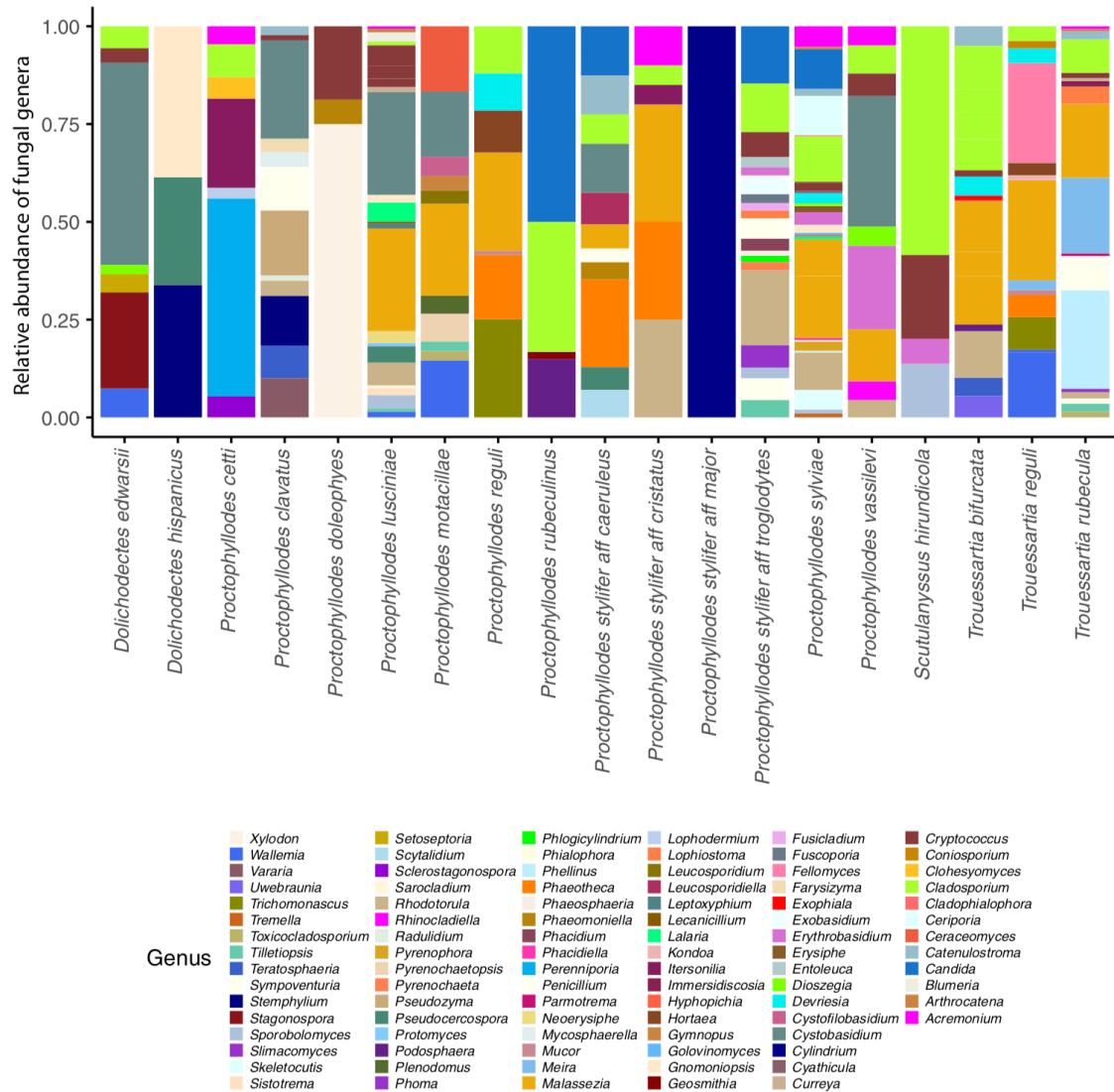
FIGURE 3 Stacked bar plots of the bacterial genera retrieved in the molecular analyses of mite species. Low abundance taxa (<2%) were not shown for illustrative purposes

TABLE 2 Prevalence and abundance (mean; minimum–maximum) statistics from the 30 most prevalent fungal and bacterial genera retrieved by DNA metabarcording. The three genera which were, on average, most abundant for each taxon, are asterisked and highlighted in bold. Relative abundance was calculated as the % of sequences of the given genus in those samples where the genus was found

Fungi	Prevalence (% of samples)	Relative abundance (% sequences within samples)	Bacteria	Prevalence (% of samples)	Relative abundance (% sequences within samples)
Cladosporium	63	17; 2-62	Sphingomonas	88	12; 5-33
Toxicocladosporium	63	26; 2-89	Acinetobacter	71	18; 5-66
Aureobasidium	53	26; 2-70	Pseudomonas	71	14; 5-50
Cryptococcus	42	6; 2-11	Sediminibacterium	53	10; 6-19
*Malassezia	42	31; 3-94	Brevundimonas	47	11; 6-18
Penicillium	42	11; 2-43	Escherichia-Shigella	41	7; 5-12
Rhodotorula	32	7; 2-21	Staphylococcus	41	14; 5-35
Acremonium	26	9; 2-18	Methylobacterium	35	8; 6-12
Catenulostroma	26	13; 3-37	Massilia	29	10; 6-21
Devriesia	26	7; 2-14	*Bartonella	24	42; 6-90
Erysiphe	26	23; 7-76	Blastomonas	24	9; 5-14
Pleurotus	26	8; 2-13	Streptococcus	24	10; 6-13
Alternaria	21	13; 6-18	Bradyrhizobium	18	6; 5-7
Aspergillus	21	10; 2-29	Corynebacterium_1	18	7; 7-7
Beauveria	21	8; 4-11	Lactobacillus	18	8; 6-10
Erythrobasidium	21	10; 2-22	Moraxella	18	8; 5-11
Sporobolomyces	21	5; 2-7	12up	12	11; 8-13
*Talaromyces	21	30; 3-98	Actinomycetospora	12	5; 5-6
Dioszegia	16	3; 3-4	Bosea	12	7; 6-9
Golovinomyces	16	13; 2-26	Chryseobacterium	12	6; 6-6
*Meira	16	47; 5-73	*Enterococcus	12	40; 14-57
Phaeotheca	16	21; 18-27	Alicyclobacillus	6	12; 12-12
Pseudocercospora	16	15; 12-21	Anaerococcus	6	7; 7-7
Stagonospora	16	11; 2-27	Aquabacterium	6	6; 6-6
Tilletiopsis	16	6; 3-8	Arcicella	6	6; 6-6
Arthrocatena	11	3; 3-3	Bacteroides	6	20; 20-20
Claviceps	11	4; 3-6	*Buchnera	6	41; 41-41
Debaryomyces	11	17; 4-29	Cloacibacterium	6	9; 9-9
Exobasidium	11	11; 9-14	Duganella	6	8; 8-8
Farysizyma	11	8; 5-12	Dyadobacter	6	10; 10-10
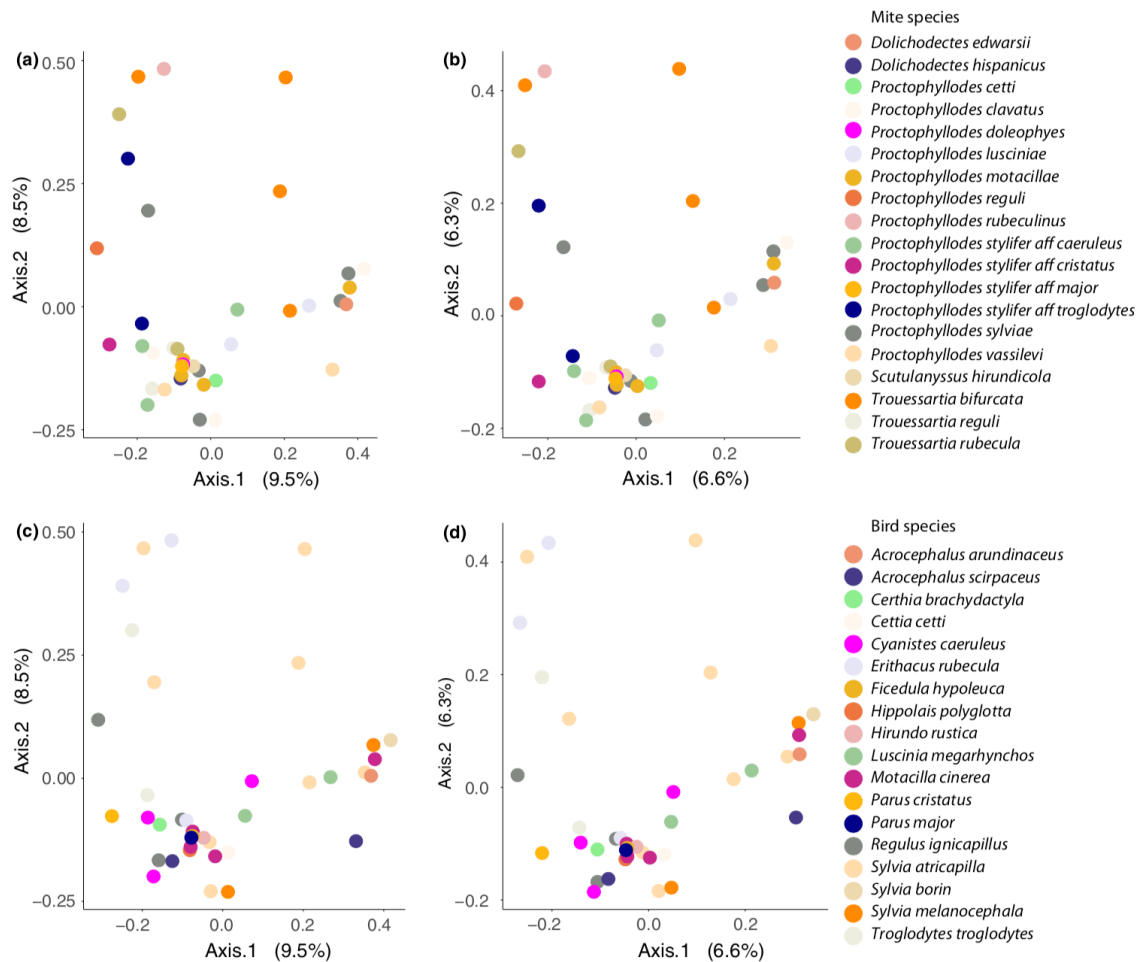
FIGURE 4 Principal coordinates analysis (PCoA) of bacterial communities of feather mite infrapopulations: First row, samples coloured by mite species and (a) based on Bray–Curtis and (b) Jaccard distances, respectively; Second row, samples coloured by bird species and c) based on Bray–Curtis and (d) Jaccard distances, respectively. OTUs counts were scaled to the smallest library following McMurdie & Holmes (2014) and Denef et al. (2016).

We found fungal DNA in all infrapopulations except one (Table S2). Overall, we retrieved a high diversity of fungal species, which was much higher in the mite samples compared to the external samples (See Material and Methods above, Figure S5). Fungal species retrieved from mite samples mostly belonged to the phyla Ascomycota and Basidiomycota, with Ascomycota being the most represented (Figure S4). At the genus level, the most prevalent were *Cladosporium*, *Toxicocladosporium* and *Aureobasidium* (Table 2, Figures 5 and S6).

On the other hand, Meira, Malassezia and Talaromyces were the most abundant fungal genera when present (Table 2, Figures 5 and S6). Interestingly, we retrieved genera for which keratinolytic activity is known, such as *Cladosporium*, *Acremonium*, *Malassezia*, *Penicillium* and *Phoma*.

FIGURE 5 Stacked bar plots of the fungal genera retrieved in the molecular analyses of mite species. Low abundance taxa (<2%) were not shown for illustrative purposes.

PERMANOVAs showed significant differences in fungal composition between mite species (51% variance, $F = 1.18$, $p = .027$) and bird species (49% variance, $F = 1.21$, $p = .016$). Nonetheless, dispersion analyses (see Methods) revealed different levels of dispersion

between mite species (F = 9.22, p = .004) and between bird species (F = 9.36, p = .002), suggesting the need for a detailed inspection of the within-species variance. By doing so, principal coordinates analyses as well as stacked bar plots at the individual level within species showed no apparent consistency of fungal profiles either within mite or bird species (Figures 6 and S6).
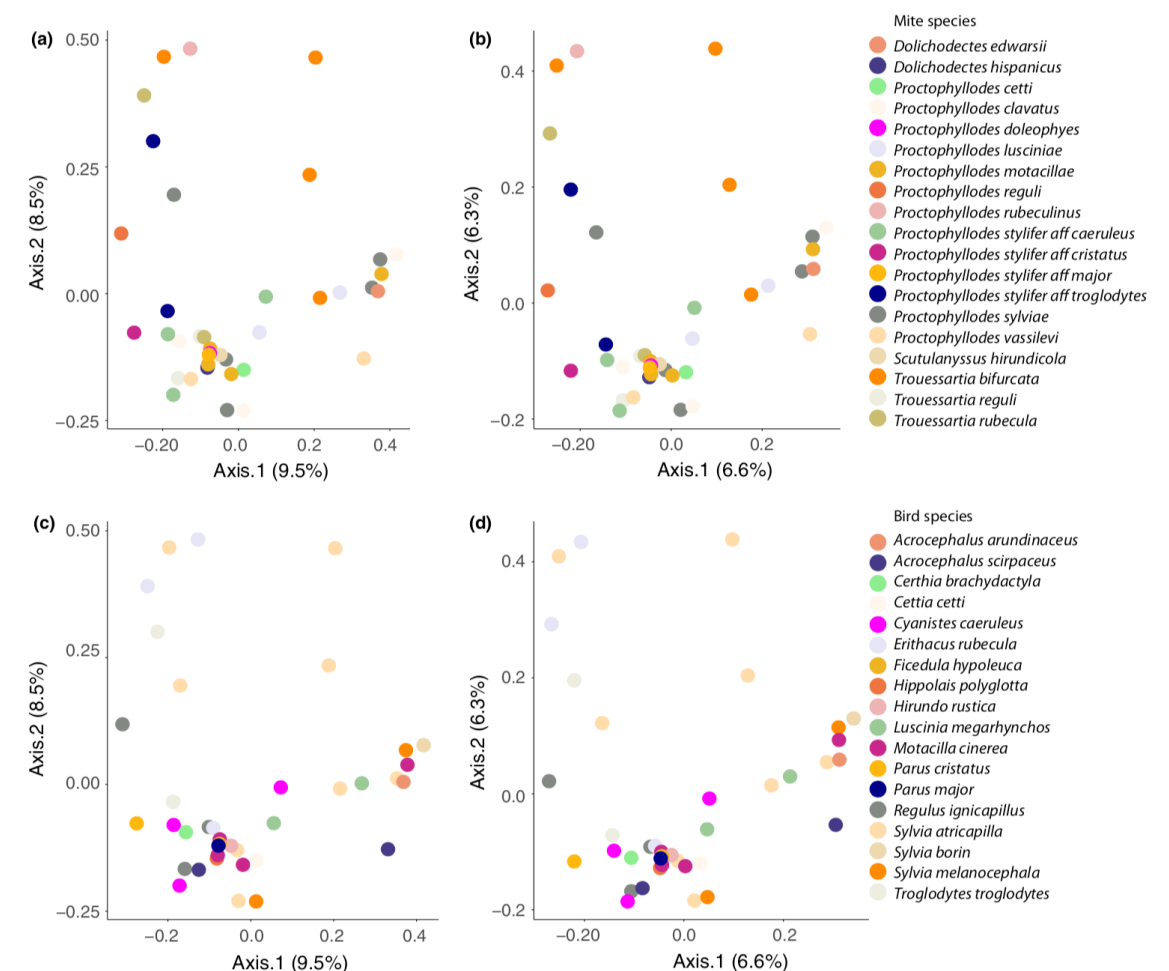
FIGURE 6 Principal coordinates analysis (PCoA) of fungal communities of feather mite infrapopulations: First row, samples coloured by mite species and (a) based on Bray–Curtis and (b) Jaccard distances, respectively; second row, samples coloured by bird species and (c) based on Bray–Curtis and (d) Jaccard distances, respectively. OTUs counts were scaled to that of the smallest library following McMurdie & Holmes (2014) and Denef et al. (2016).

Plant DNA was only sequenced from two infrapopulations (of 34) from two mite species inhabiting two different bird individuals. The first infrapopulation from which plant DNA was recovered belonged to *Proctophyllodes sylviae* Gaud, 1957 from the Blackcap *Sylvia*

atricapilla (Linnaeus, 1758). Plant OTUs retrieved matched to *Polygala teretifolia* Thunb. (99.7% pairwise similarity; grade 88.6%), *Citrus clementine* hort. (two OTUs: 92.8, 98.4% pairwise similarity; grade 96.4, 99.2%), *Daphne laureola* L. (94.9% pairwise similarity; grade 93.2%) and *Digitalia ciliaris* (Retz.) Koeler. (96.5% pairwise similarity; grade 96%). The second infrapopulation belonged to *Trouessartia bifurcata* (Trouessart, 1885) also from a *Sylvia atricapilla* host, in which the single OTU retrieved matched to *Quercus* sp. (95.5% pairwise similarity; grade 97.7%).

Discussion

In this study, by analysing the diet of feather mites using both DNA metabarcoding and microscopy-based methods, we investigated the long-standing question of the nature of the interaction between birds and feather mites. Fungi and potentially bacteria (see below) were revealed as the main recognizable food resources for feather mites, while diatoms and plant matter appeared as rare food resources. Similarly, Dubinin (1951) examined the guts of 18,735 specimens of *Freyana* spp. (Freyanidae) from waterfowl and found diatoms in only 135 of them (0.72%). Importantly, we did not find visual or DNA evidence of feather mites feeding upon bird resources (e.g., blood, skin) other than likely uropygial gland oil (see Materials and Methods), in spite of using primers suitable for amplifying degraded bird DNA. We observed no obvious feather filaments in our microscopy analysis, but this and our molecular study would not have been able to identify tiny (non-DNA-bearing) fragments of feathers, which have been occasionally reported in microscopy studies. The chelicerae of vane-dwelling feather mites do not seem capable of cutting or tearing intact feathers, so if the tiny fragments we observed in the guts are indeed feather fragments, they would likely be ingested along with other loose material. In addition, we found a high prevalence of both keratinophilic and pathogenic fungal taxa (e.g., *Cladosporium*, *Penicillium*, Al Rubaiee et al. 2017; Friedrich et al. 1999; Gunderson, 2008; Marchisio et al. 1991; Nwadiaro et al. 2015) in feather mite guts. Whether the quantities of bacteria and fungi eaten by feather mites are enough to

increase host fitness requires further study. Altogether, our results support previous evidence on the commensalistic–mutualistic role of vane-dwelling feather mites (Blanco et al. 1997, 2001; Galván et al. 2012; Proctor, 2003; Walter & Proctor, 2013a, b, c). Thus, vane-dwelling feather mites probably should no longer be considered to be parasites of birds (e.g., Harper, 1999) but rather commensalists–mutualists. This does not apply to the few taxa of quill-dwelling feather mites that clearly feed on feather pith (e.g., Ascouracaridae) or those that live on or in the epidermis of the host (e.g., Dermationidae, Epidermoptidae) (Gaud & Atyeo, 1996; Proctor, 1999). Additionally, whether uropygial gland oil constitutes an important food resource for feather mites remains unanswered from our data (Pap et al. 2010) and should be studied using more sensitive methods (e.g., HPLC, histological staining analysis). Indeed, should uropygial gland oil be beneficial for birds, a large number of mites feeding upon this resource might have a detrimental effect on host fitness (Blanco et al., 2001). However, a recent review concluded that is not even clear how or if uropygial gland oil affects bird fitness (Moreno-Rueda, 2017). In the light of our findings, previous occasional documentation of unhealthy birds with high numbers of vane-dwelling feather mites (e.g., Atyeo & Gaud, 1979) could be reinterpreted as birds in poor condition providing more food resources to feather mites (e.g., fungi and bacteria, which may be directly or indirectly related with host' health status, Blanco et al. 2001; Soler et al. 2012). It may also be that birds in poor condition preen less, which could in turn impact the abundance of feather mites if they are susceptible to removal by preening activities. However, it remains the possibility that feather mites have an effect on host fitness by removing preen gland oil, by potential aerodynamic costs of harbouring large amounts of mites and by indirect effects on host fitness mediated by other ectoparasites (e.g., the occasional ingestion of feather mites by feather lice which may indirectly increase the cost of parasitism of feather lice).

The possibility that symbiont species might be at risk of extinction (e.g., Carlson et al. 2017; Rózsa & Vas, 2014) suggests the need for a rapid integration of

this knowledge into bird-related practices, conservation programmes. Also, our results suggest that further suggest that further studies of birds in farms, zoos and the pet trade are needed, where traditionally feather mites were viewed as parasites, with birds provided with treatment using acaricides (e.g., Alekseev, 1998; Salisch, 1989). This practice not only has the downside of monetary expense because of the use of acaricides, but could also result in the loss of the potential services provided by feather-cleaning mites, as our results suggest.

Analyses of the bacterial and fungal DNA found in the guts of feather mites revealed a high diversity of taxa that were not structured by host or by mite species (Figures 4, 6 and S6-S9). This suggests trophic opportunism of mites (da Silva et al. 2015; Kent & Burtt, 2016), which may graze upon whatever food resources might be available at the time. This opportunistic "feather-cleaning" feeding behaviour is also supported by the large amount of unidentifiable items we found in the guts and by the higher abundance and diversity of fungi found in the mite samples in comparison with the external samples (e.g., Figures S3 and S10). Overall, many other species of sarcoptiform mites, including many free-living Astigmata, are functionally defined as fungivore–microbivore–detritivores (e.g., Pyroglyphidae and most oribatid mites, Walter & Proctor 2013a, b), and our results also support this classification for feather mites. In fact, our results are in large agreement with previous studies on microbes found in other mite species (Chaisiri et al. 2015; Hubert et al. 2012), where strong evidence has been found for the utilization of bacteria as a food source in free-living astigmatan species (Erban & Hubert, 2008, 2010; Hubert et al. 2014, 2016). In these studies, microbiomes composed of highly diverse taxa in low abundance have been interpreted as evidence for microbivory. In contrast, microbiome profiles showing a low diversity of highly abundant taxa are interpreted as evidence of symbiotic or pathogenic bacterial species (Hammer et al. 2017; Hubert et al. 2016). In this way, the prevalence–abundance patterns of the bacteria found here (Table 2) suggest a combination of bacteria used as food resource (mostly environmental-associated

genera, which were more prevalent but less abundant, for example, *Sphingomonas* and *Acinetobacter*; Table 2) and of potentially symbiotic, commensalistic or pathogenic bacteria (less prevalent but much more abundant when present, for example, *Bartonella*, *Enteroccocus*; and the primary endosymbiont, *Buchnera*; Table 2).

Lack of a stable "microbiome" across different individuals of a given species has been found in other organisms with a nutritionally broad diet (Shapira, 2016). In contrast, species with highly biased diets, such as lice feeding on bird feathers (mainly keratin) or termites feeding on dead wood (mainly cellulose), typically have permanent and relatively stable endosymbiotic bacteria which provide them essential vitamins or other nutritional supplements (Puchta, 1955; Ohkuma, 2008; Perottiet al. 2009; Boyd et al. 2016; but see Hammer et al. 2017). Thus, our results suggesting the lack of a stable microbiome at the mite species level add support to the hypothesis of a generalist fungivore–microbivore–detritivore diet for the feather mites reported here, instead of these resources being taken as a by-product of a diet based mostly on uropygial oil (Engel & Moran, 2013; Sanders et al. 2017; Shapira, 2016). In fact, in 42% of the mites in which we detected any food resource, we did not see any oil globules (but see Materials and Methods) also suggesting that resource intake does not depend on oil ingestion.

A further understanding of the multilayered hologenome (i.e., to distinguish between stable–unstable, adapted–unadapted bacterial taxa, Shapira, 2016) through large-scale microbiome-oriented studies will help in disentangling the role of these potentially symbiotic or pathogenic bacteria of feather mites. Furthermore, whether feather mites select among available food resources (fungal preferences have been found in free-living fungivorous Astigmata, Hubert et al. 2003, 2004) or do not need to rely on bacterial symbionts requires further experimental study. Lastly, a hypothesis of an "external-rumen" mode of feeding, in which mites ingest predigested food (by

bacteria), has been also supported in free-living astigmatan mites (Hubert et al. 2014, 2016) and would be also compatible with our results.

Feather mite species are relatively host-specific and (presumably) host-specialized symbionts that appear to have relatively low levels of switching to new host species (Doña et al. 2017; Doña et al. 2017; Gaud 1992; Klimov et al. 2017; Matthews et al. 2018). These switches mostly involve closely related hosts, but major-host switches (e.g., between bird orders) have been revealed as a major driver of their diversification (Doña et al. 2017). As for many other host–symbiont systems (Clayton et al. 2016; Nylin et al. 2017), understanding the (co)eco-evolutionary scenario of host-switching in this host–symbiont system is still in its infancy. However, the likely opportunistic diet of feather mites reported here suggests that host-switching of feather mites would not be constrained by the extrinsic nutritional resources available on the new host (but it may be, for example, by feather morphology or by the bird preening efficiency; Clayton et al., 2005). Uropygial gland oil composition, however, differs between birds (Soini et al. 2013); and whether mites are specialized to host oil is unknown, and requires further study. Nevertheless, the fact that different bird species can harbour contrasting (and consistent) abundances of feather mites (Diaz-Real et al. 2014; Doña et al. 2015) suggests that, among others factors, the abundance of food resources for feather mites could strongly differ between bird species, but this also needs additional research.

Overall, this study supports the hypothesis that the interaction between birds and vane-dwelling feather mites involves commensalism or mutualism, with feather mites acting as feather-cleaners of birds. This opens the possibility of studying bird-feather mites as an interesting case study of defensive symbiosis (Hopkins et al. 2017). Further experimental research is needed to unravel the likely context-dependent (possibly even occasionally parasitic) relationship between vane-dwelling feather mites and birds (Blanco et al. 2001). In particular, future studies should investigate the following. (i) Using appropriate and sensitive methods such as HPLC,

test whether uropygial gland oil is part of the diet of feather mites. A comparative exploration of the diet of feather mites inhabiting birds with vestigial uropygial gland that produce powder down would be also useful. If uropygial oil is a large component of vane-dwelling feather mites, it would be then important to test whether removal of the oil affects bird fitness. (ii) Investigate whether the diet of feather mites differs along the annual cycle of birds (e.g., migration, moult). (iii) Examine the potential aerodynamic costs of harbouring different quantities of feather mites. (iv) Determine effects of feather mites on host fitness as mediated by other ectosymbionts (e.g., feather lice). (v) Test whether an experimental increase in feather mites' abundance increases, decreases or has no overall effect on host fitness. Lastly, (vi) examine whether experimental variation in feather mites abun- dance has a context-dependent (e.g., under different environmental conditions) effect on host fitness over time.

Chapter 6.

Cophylogenetic analyses reveal extensive host-shift speciation in a highly specialized and host-specific symbiont system

Abstract

Host-shift speciation and cospeciation are the two major processes driving symbiont macroevolutionary diversification. Cospeciation is expected to be frequent in vertically transmitted and host-specific symbionts, and leads to congruent host-symbiont phylogenies. However, the cophylogenetic dynamics of many groups of highly specialized host-specific symbionts is largely unstudied. Thus, the relevance of cospeciation vs. host-shift spe- ciation remains largely unknown. Here, we investigated this question by performing the largest cophylogenetic study of feather mites to date, using both distance and event-based cophylogenetic methods. For these analyses, we inferred phylogenies based on all protein coding genes of the mitochondrial genome of *Proctophyllodes* and *Trouessartia* feather mite species living on European passerine birds. Results show high incongruence among bird and feather mite phylogenies, because of extensive host-switching. We conclude that host-shift speciation, rather than cospeciation, may be the main driver of symbiont diversification even for highly specialized symbionts with low host-switching potential.

Introduction

Understanding the evolutionary diversification of symbiont species remains a challenge, with most symbiont groups unstudied (Althoff et al., 2014; Ricklefs et al., 2014; Clayton et al., 2016), albeit with major implications for relevant areas such as emerging infectious diseases (Hoberg and Brooks, 2015), biological invasions (Dunn, 2009; Traveset and Richardson, 2014) and climate change (Carlson et al., 2013). Macroevolutionary events, such as host-shift speciation, cospeciation, sorting (i.e. extinction and "missing the boat"), and duplication events are the drivers of most of the current diversity of symbiont species (Janz, 2011; de Vienne et al., 2013; Clayton et al., 2016). Cospeciation (symbiont speciation following host speciation) and host-shift speciation (symbiont speciation following a host-switch) are the main alternative modes involved in generating diversity in most symbiont lineages (de Vienne et al., 2013; Clayton et al., 2016). However, the relative importance of these processes is highly variable among host-symbiont systems, and although strongly linked to particular ecological and evolutionary traits (Jousselin et al., 2009; Ricklefs et al., 2014; Clayton et al., 2016), we still lack a solid understanding of which scenarios favor one mechanism over the other.

Host-switches can lead to diversification (i.e. host-shift speciation) when a symbiont species moves to a new host species, successfully colonizes it, and eventually speciates (Johnson and Clayton, 2004; Giraud et al., 2010; Janz, 2011; de Vienne et al., 2013). Host-switches are more likely in symbionts with high dispersal potential (Clayton et al., 2016), such as those with free-living stages (e.g. Platyhelminthes; Braga et al., 2015), those that use vectors for transmission (e.g. avian malaria parasites; Ricklefs et al., 2004, 2014), or ectosymbionts dispersing attached to other symbionts (i.e. phoresis, e.g. Brueelia feather lice; Johnson et al., 2002; Bush et al., 2016). Host-switches are pervasive and are considered to be the main driver of symbiont diversification (Janz, 2011; de Vienne et al., 2013), to the extent of being reported as the most relevant drivers of the diversification process, in 93% of the cophylogenetic studies reviewed by de Vienne et al. (2013).

However, cospeciation is expected to be the main driver of diversification in vertically transmitted and host-specific symbionts, in which symbiont reproduction is strongly tied to host reproduction (McCoy et al., 2003; Thompson, 2005; Clayton et al., 2016). Examples of systems with extensive cospeciation include viruses, bacteria, nematodes, and mites

(reviewed in Clayton et al., 2016). However, the cophylogenetic dynamics of most of highly specialized, host-specific symbionts is largely unstudied, so the relevance of cospeciation vs. host-shift speciation among host-specific symbionts remains an open question.

Feather mites (Acari: Astigmata: Analgoidea and Pterolichoidea), the most diverse and abundant ectosymbionts of birds, are an interesting model system to tackle this question (Gaud and Atyeo, 1996; Proctor and Owens, 2000; Proctor, 2003; Dabert, 2004; Doña et al., 2016). Plumage-dwelling feather mites (hereafter, feather mites) are host-specific and highly specialized mites that spend their entire life-cycle on their host's flight feathers (Proctor, 2003). Feather mites cannot survive off of their host (Dubinin, 1951; Proctor, 2003), and have developed several adaptations for this obligate lifestyle: flattened bodies, sucker-like pretarsi (ambulacra), and various clasping and seizing mechanisms on their bodies and legs to avoid becoming dislodged out of the host (Mironov, 1999), and also behavioral adaptations such as avoiding feathers that are about to be molted (Jovani and Serrano, 2001). Feather mites only leave the host during transmission, mainly when they pass from parents to offspring (i.e. vertical transmission) (Mironov and Malyshev, 2002; Doña et al., 2017). In addition, feather mites are not transmitted by phoresy (Jovani et al., 2001), as opposed to some dermicolous epidermoptid mites, which do (Jovani et al., 2001; Proctor, 2003). Feather mites are thus an excellent highly specialized, vertically transmitted symbiont model to test whether (as expected) cospeciation is predominant over host-shift speciation (Proctor and Owens, 2000; Dabert, 2004; Agosta et al., 2010). In fact, studies of feather mites of the families Avenzoariidae (Analgoidea) and Freyanidae (Pterolichoidea) associated with non-passerine avian orders support cospeciation as the dominant process (Mironov and Dabert, 1999; Ehrnsberger et al., 2001; Dabert et al., 2001). However, most feather mite taxonomic groups remain unstudied in a cophylogenetic context, and there are no studies using the recently developed analytical and methodological tools.

Here, we present the largest cophylogenetic study of feather mites to date by using both distance and event based cophylogenetic methods to study species of the genera *Proctophyllodes* and *Trouessartia* living on European passerine birds. *Proctophyllodes* and *Trouessartia* are the most speciose genera among all feather mites, and are generally asso-ciated with passerine birds (Atyeo and Braasch, 1966; Santana, 1976; Mironov, 2012; Doña et al., 2016). Within Proctophyllodes analyses we also included two species from the genus

Monojoubertia and one from *Joubertophyllodes*. This is because their genus status is unsupported by recent phylogenetic studies, suggesting they belong to the genus *Proctophyllodes* as part of the glandarinus and pinnatus species groups, respectively. Hereafter they are treated as *Proctophyllodes* for the sake of text clarity (Knowles and Klimov, 2011; Klimov et al., 2017a).

For the analyses, we generated phylogenies based on all protein coding genes of the mitochondrial genome. Our results show high incongruence among bird and feather mite phylogenies because of extensive host-switching. We conclude that host-shift speciation, rather than cospeciation, may be the main driver of symbiont diversification even for highly specialized symbionts with low host-switching potential.

Materials and methods

DNA libraries preparation

Illumina libraries for 64 feather mites were constructed using the DNA extracts from Doña et al. (2015a), covering a great fraction of mite species from these genera in European passerines; see Table S1 Supporting information in Doña et al. (2015a) for voucher details. A total amount of 1.0 μg genomic DNA per sample was used as input for the DNA sample preparation carried out at Novogene (China). Sequencing libraries were generated using Agilent SureSelect Human All ExonV5 kit (Agilent Technologies, CA, USA) following the manufacturer's recommendations. Index codes were added to each sample. Briefly, fragmentation was carried out by hydrodynamic shearing system (Covaris, Massachusetts, USA) to generate 180–280 bp fragments. Remaining overhangs were converted into blunt ends via exonuclease/ polymerase activities and enzymes were removed. After adenylation of 3′ ends of DNA fragments, adapter oligonucleotides were ligated. DNA fragments with ligated adapter molecules on both ends were selectively enriched in a PCR reaction. After the PCR reaction, libraries were hybridized with Liquid phase with biotin labelled probe, then magnetic beads with streptomycin were used to capture exons. Captured libraries were enriched in a PCR reaction to add index tags to prepare for hybridization. Products were purified using AMPure XP system (Beckman Coulter, Beverly, USA) and quantified using the

Agilent high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina, San Diego, USA) according to the manufacturer's instructions. Then, we applied whole-genome shotgun sequencing using the Illumina HiSeq 4500, generating 150 bp paired-end reads. In total, the sequencing of the 64 libraries produced 64 Gb of data (~1 Gb per library).

Mitochondrial genome assembly

FASTQ files were quality-trimmed at a base call error probability limit of less than 0.05 in *Geneious 9.1.5* (http://www.geneious.com, Kearse et al., 2012). Mitochondrial genomes were assembled by using the quick option of *MITObim* (Hahn et al., 2013). For each mite library, we ran two assemblies: 1) using the COI sequence from the same individual mite (Doña et al., 2015a) as starting seed; and 2) using a feather mite mitochondrial genome as reference. *Trouessartia kratochvilli* was the first genome assembled by the first approach, and was used as reference for the second approach. In addition, we checked that there were no differences in assembly success depending on the reference used. After running both assemblies, we visually inspected the genomes, and only kept the longest assembled contig for further analyses (Table S1, Supporting information).

Assembly quality check

All mitochondrial genomes were annotated using *MITOS* (Bernt et al., 2013). We then remapped raw reads from each library against the corresponding annotated genome to verify that reads mapped correctly. We used the *Geneious* read mapper with Medium-Low sensitivity and default parameters. The results of the map-to-reference analyses were inspected manually. Feather mite mitochondrial genomes have the same 13 protein coding genes (PCGs) as dust mites (Dermauw et al., 2009) and other arthropods. In this study, all PCGs were assembled, but only those individual PCGs assembled with high coverage (~20X) were kept for later analyses. Lastly, we translated each gene sequence into amino acids (into each of six possible reading frames) and removed those sequences with stop codons.

Phylogenetic analysis

We aligned each gene separately from each feather mite genus using *MAFFT v7.222* (Katoh et al., 2002). Taxa with less than 3,000 bp (n = 24) were removed from the alignment for the backbone phylogenetic tree (see below) because of their presumed lower phylogenetic information content as a result of extensive missing data (Gómez-Rodríguez et al., 2015). We then made an additional COI alignment to posteriorly place the initially removed taxa into the tree based now only on COI sequences from Doña et al. (2015a) (see below) (Zhou et al., 2016). All the alignments were trimmed with *Trimal v1.2* (Capella-Gutiérrez et al., 2009) and checked by eye in *Geneious*. We also checked for saturation of our marker (Fig. S7). We concatenated the 13 gene alignments and used this matrix as input for *Partition Finder v1.1.1* (Lanfear et al., 2012) to find the optimal partitioning scheme and substitution models of each partition. The Bayesian information criterion (BIC) with linked branch lengths and the greedy algorithm were used to search for the best-fitting partitioning model.

We inferred the maximum-likelihood backbone tree using the edge-proportional partition model in *IQ-TREE* (Nguyen et al., 2015; Chernomor et al., 2016). We used this tree as backbone constraint tree to place the taxa for which only COI sequences were available by using the '-r' option in *RAxML 8.1.2* (Stamatakis, 2014). *Proctophyllodes* and *Trouessartia* trees were inferred using *Scutulanyssus hirundicola* (Pteronyssidae, SRA accession number: SRR5319233) and *Gabucinia delibata* (Gabuciniidae, SRA accession number: SRR5319261) respectively as outgroups (Klimov and O'Connor, 2008; Dabert et al., 2010; Klimov and O'Connor, 2013).

Cophylogenetic analysis

We analyzed the cophylogenetic dynamics using distance-based and event-based methods (Sweet et al., 2016; Sweet and Johnson, 2016). Before the cophylogenetic analyses, we pruned the feather mite phylogeny so that each species was represented by a single tip, following the species criterion resulting from Doña et al. (2015a). We also removed the outgroup taxa. For the taxonomically uncertain species of the pinnatus-group (*Proctophyllodes pinnatus*, *P. neopinnatus*, *P. serini*, and *P. troncatus*; see Doña et al., 2015a for details), we analyzed the among-species p-distances using all PCGs. Species in the

pinnatus-group showed < 1.5% among-species divergence, far less than the 9% mean smallest interspecific distances found in Doña et al. (2015a, b) for feather mites. Thus, we collapsed these species into a single tip, because improperly delimited species can overestimate cospeciation events (Refrégier et al., 2008). To obtain a host phylogenetic tree, we downloaded trees for relevant avian host taxa from the BirdTree database (Jetz et al., 2012, http://birdtree.org). In particular, we download 1,000 trees from the Hackett backbone tree (only sequenced species, Hackett et al., 2008) and then summarized those trees by computing a single 50% majority-rule consensus tree using *SumTree v 4.1.0* in *DendroPy v4.1.0* (Sukumaran and Holder, 2010, 2015) following Rubolini et al. (2015).

For distance-based cophylogenetic analyses, we used *ParaFit* (Legendre et al., 2002) and *PACo* (Balbuena et al., 2013) to assess overall congruence between host and symbiont phylogenies. In addition, these methods allow for the quantification of the relative contribution of individual host-symbiont associations to the overall congruence. *ParaFit* assesses whether symbionts are randomly associated with their hosts, whereas *PACo* studies the dependence of the symbiont phylogeny on the host phylogeny through a residual sum of square goodness-of-fit test. We ran *ParaFit* for 100,000 permutations using the *parafit* function implemented in the *R* package *APE v3.5* (Paradis et al., 2004) with Cailliez correction for negative eigenvalues and testing for the contribution of each individual link using the ParaFitLink1 and ParaFitLink2 tests. We corrected individual link p-values using the Bonferroni correction. A significant link suggests that particular host- symbiont association contributes to the global congruence between the host and symbiont trees. We also ran *PACo* for 100,000 permutations with the *APE* and *VEGAN v2.4.1R* packages (Oksanen et al., 2016), and used the jackknife method to estimate the importance of each individual link to the overall sum of squares score.

For the event-based analysis, we used *Jane v4* (Conow et al., 2010). Jane uses a Genetic Algorithm (GA) to reconstruct the optimal (lowest cost) set of evolutionary events (cospeciation, host switch, etc.) that reconciles host and symbiont trees based on a priori event costs. For the GA parameters we used 100 generations and a population size of 500 (Conow et al., 2010), and default event costs (0 cospeciation, 1 duplication, 2 duplication and host switch, 1 loss, and 1 failure to diverge). These costs are appropriate for obligate symbionts (Conow et al., 2010; Hamerlinck et al., 2016), and the higher cost for host

switches is conservative against an over-estimation of that event. In addition, we used the time zone constraint option implemented in Jane. This option allows the possibility of limiting ''host-switch'' events to certain time periods (i.e. time zones) in the respective trees to force them to occur among historically co-occurring lineages. This is done without having a time-informed tree, but having time information for some nodes, which is used to delimit the time zones (Conow et al., 2010). We searched for all the information about the *Proctophyllodes* and *Trouessartia* taxa studied here available at the TimeTree repository (Hedges et al., 2006), only finding information for *Proctophyllodes* taxa. In particular, we dated the *Proctophyllodes stylifer - Monojoubertia microphylla* node from Dabert et al. (2010). Subsequently, two time zones were defined for the *Proctophyllodes* analysis, one with all the speciation events before this node and another with all the events after. Once we found the optimal solution, we randomized the tip mappings 999 times to determine if the sum of costs needed to reconcile the two phylogenies obtained by the optimal solution was lower than expected by chance. A significant result from this statistical test occurs if the randomization procedure indicates our best score from the data is lower than by chance, and would indicate some level of congruence between the host and symbiont phylogenies (Sweet and Johnson, 2016). Lastly, to evaluate how uncertainty in our mite' phylogenetic reconstruction is biasing our cophylogenetic analyses, we repeated for both genera our distance-based and event-based analyses but using a 50% majority-rule consensus tree for the mites.

Results

The final concatenated matrix of the backbone tree of *Proctophyllodes* species contained 27 species and 9,525 bp (3,890 informative sites) with a 95% per position mean completeness. For *Trouessartia* species, we obtained a matrix of 12 species and 9,681 bp (3,840 informative sites) with a 97% per position mean completeness (Table S1, Supporting information). The maximum likelihood backbone trees were well supported. For *Proctophyllodes*, 80% of the nodes were supported by greater than 75% bootstrap and for *Trouessartia*, all of the nodes were supported by greater than 75% bootstrap (Table S1, Fig. 1). The overall support decreased, mostly at terminal nodes, when including species for which only COI was available. For these expanded trees *Proctophyllodes* had 60% of nodes above 75% bootstrap and *Trouessartia* had 64% of nodes above 75% bootstrap (Fig. 1, Figs. S3 and

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S4, Supporting information). Nevertheless, our trees are largely in agreement with previous studies covering a much smaller fraction of the species studied here. The total expanded trees were comprised by 44 species of *Proctophyllodes* (see Introduction) and 15 of *Trouessartia*.

Proctophyllodes and *Trouessartia* phylogenies showed little obvious congruence when compared to their host trees (Fig. 1). Nevertheless, *ParaFit* and *PACo* distance-based tests detected a significant level of overall congruence between both mite genera and their hosts ($P < 0.01$). ParaFitLink1 tests showed that only 13 host-associations from the Proctophyllodes tree were significantly contributing to the global score after correcting for multiple tests ($\alpha = 0.05$) (Fig. S1, Supporting information). *PACo* individual jackknife link test also recovered 13 host-symbiont interactions with the 95% confidence intervals of their squared residuals lower than the median global squared residual (Fig. S1, Supporting information). The analyses using the 50% majority-rule consensus tree showed almost identical results. ParaFit and PACo distance-based tests also detected a significant level of overall congruence between both mite genera and their hosts ($P < 0.01$). The only differences were that ParaFitLink1 tests showed 12 instead of 13 host-associations significantly contributing to the global score after correcting for multiple tests ($\alpha = 0.05$), and that *PACo* recovered 10 versus 13 host-symbiont interactions with the 95% confidence intervals of their squared residuals lower than the median global squared residual. For Trouessartia species, only two host-associations were recovered as significant by *PACo* and zero by ParaFitLink1 (Fig. S2, Supporting information). The results of analyses done using the 50% majority-rule consensus tree were identical. For both genera, ParaFitLink2 tests *p*-values were identical to those using ParaFitLink1. The event-based method also recovered little congruence between symbiont and host trees, with a small fraction of host nodes inferred to be involved in a cospeciation event (Fig. S3, Supporting information). Only the *Proctophyllodes* reconstruction had an observed cost (sum of all events) that was significantly lower than by chance (71; $P < 0.01$). Nevertheless, for this genus, optimal solutions indicated that the number of inferred host-switches (30) was over twice the number of inferred cospeciation events (13) needed to reconcile host and symbiont phylogenetic trees (Table 1, Fig. S3, Supporting information). Additionally, the percentage of host nodes showing cospeciation events with their mites was low (30%; Fig. S3, Supporting information). For the *Trouessartia*

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reconstruction, the cost (19) was not lower than expected by chance (P = 0.11). The proportions of reconstructed events were similar to those recovered for *Proctophyllodes*, i.e., requiring more host-switches (nine) than cospeciation events (five; Fig. S4, Supporting information; Table 1). As for *Proctophyllodes*, the percentage of host nodes showing cospeciation events with their mites was low (35%, Fig. S4, Supporting information). The analyses done using the 50% majority-rule consensus tree showed highly congruent results. Optimal solutions inferred more host-switches (32 instead 30, Table 1) and less cospeciation events (11 versus 13 cospeciation, Table 1).

Table 1 Results for the birds-feather mites cophylogenetic analysis with Jane v4. Values in parentheses show the results of the analysis done using a 50% majority-rule consensus tree.

	Cospeciations	Duplications	Duplications and host- switches	Losses	Failures to diverge
Proctophyllodes	13 (11)	0 (0)	30 (32)	11 (1)	0 (0)
Trouessartia	5 (5)	0 (0)	9 (9)	1 (1)	0 (0)

a)

Proctophyllodes weigoldi — Parus ater
Proctophyllodes vassilevi — Parus major
Proctophyllodes vitzthumi — Parus caeruleus
Proctophyllodes rubeculinus — Remiz pendulinus
Proctophyllodes lusciniae — Acrocephalus scirpaceus
Proctophyllodes doleophyes — Acrocephalus schoenobaenus
Proctophyllodes caulifer — Sylvia borin
Proctophyllodes cotyledon — Sylvia atricapilla
Proctophyllodes ateri — Cettia cetti
Proctophyllodes stylifer — Sitta europaea
Proctophyllodes stylifer sp — Erithacus rubecula
Proctophyllodes reguli — Luscinia luscinia
Proctophyllodes acanthicaulus — Luscinia svecica
Proctophyllodes tenericaulus — Ficedula hypoleuca
Proctophyllodes mesocaulus — Phoenicurus ochruros
Proctophyllodes hipposideros — Phoenicurus phoenicurus
Proctophyllodes leptocaulus — Saxicola rubetra
Proctophyllodes musicus — Muscicapa striata
Proctophyllodes musicus sp — Turdus merula
Proctophyllodes clavatus — Turdus viscivorus
Proctophyllodes clavatus sp.n — Turdus philomelos
Proctophyllodes cetti — Regulus regulus
Proctophyllodes sylviae — Prunella collaris
Proctophyllodes remizicola — Prunella modularis
Proctophyllodes megaphyllus — Miliaria calandra
Proctophyllodes vegetans — Emberiza cia
Proctophyllodes miliariae — Emberiza schoeniclus
Proctophyllodes ciae — Carpodacus erythrinus
Joubertophyllodes modularis — Carduelis spinus
Proctophyllodes schoenicli — Loxia curvirostra
Proctophyllodes spini — Pyrrhula pyrrhula
Proctophyllodes poublani — Coccothraustes coccothraustes
Proctophyllodes schwerinensis — Fringilla coelebs
Proctophyllodes troncatus — Fringilla montifringilla
Proctophyllodes sp.n — Anthus pratensis
Proctophyllodes fuchsi — Anthus spinoletta
Proctophyllodes motacillae — Anthus trivialis
Proctophyllodes glandarinus — Motacilla flava
Proctophyllodes anthi — Passer domesticus
Proctophyllodes simillimus — Montifringilla nivalis
Monojoubertia microphylla — Lanius senator
Monojoubertia hemiphylla — Garrulus glandarius
Proctophyllodes volgini
Proctophyllodes macedo

b)

Trouessartia inexpectata — Sylvia undata
Trouessartia reguli — Sylvia atricapilla
Trouessartia tenuipilata — Cisticola juncidis
Trouessartia kratochvili — Locustella luscinioides
Trouessartia bifurcata — Acrocephalus arundinaceus
Trouessartia trouessarti — Riparia riparia
Trouessartia minuscula — Hirundo rustica
Trouessartia swidwiensis — Regulus regulus
Trouessartia rubecula — Luscinia luscinia
Trouessartia simillima — Erithacus rubecula
Trouessartia ripariae — Muscicapa striata
Trouessartia microcaudata — Sturnus vulgaris
Trouessartia rosterii — Estrilda astrild
Trouessartia appendiculata — Motacilla alba
Trouessartia jedlizckai

Fig. 1.

FIGURE 1 Tanglegram of birds and their associated feather mites. a) Proctophyllodes species. b) Trouessartia species. (*) nodes with ML bootstrap (BS) support ≥ 75.

Discussion

Comparisons of the phylogenies of two genera of feather mites with those for their avian hosts showed high incongruence across all methods used. For both genera, the number of host-associations contributing to overall congruence (using *ParaFit* and *PACo*) as well as the percentage of nodes showing cospeciation (using *Jane*) was very low (Fig. 1; Figs. S1 and S2, Supporting information). Tree reconciliations and event-based reconstructions recovered a high number of host-switches, typically around double that as for cospeciation events (even higher for the analyses done using a 50% majority-rule consensus tree). Host-switches are the most difficult event to reconstruct for cophylogenetic algorithms, and hence it is most relevant that we recovered so many switching events in our analyses (Charleston, 1998; Conow et al., 2010). This high preponderance of host-switches was not expected given the high level of host-specificity, the low dispersal potential of these feather mites, and previous reports of high cospeciation in Freyanidae and Avenzoariidae feather mites (Mironov & Dabert, 1999; Ehrnsberger et al., 2001; Dabert et al., 2004). Therefore, our results support that the importance of host-shift speciation in symbiont diversification may be underestimated (de Vienne et al., 2013).

Our findings are in line with expectations for symbionts that colonize new hosts by means of ecological fitting (Agosta and Klemens, 2008; Agosta et al., 2010). Although host-switches are likely to be less frequent in species with lower dispersal capabilities, such as the mites in this study (Clayton et al., 2016), they are likely to undergo strong disruptive selection after switching to a new host (Agosta and Klemens, 2008; Nyman, 2010; Janz, 2011). In part, this is precisely because of their low dispersal ability, and thus the low probability of continued gene flow between populations on the new and original host species, leading to divergence. Therefore, while likely relatively infrequent on ecological timescales (based on that feather mites are highly host-specific), host-switches may have a major impact on the evolutionary diversification of highly specialized symbionts (Johnson et al., 2006, 2011, 2012). Therefore, our results show that qualitative knowledge of the between-host dispersal potential of a highly specialized symbiont group does not necessarily accurately predict the relative role of cospeciation versus host-switching in dictating symbiont diversification. This should encourage more cophylogenetic analyses on other specialized symbionts, and quantitative studies on their actual between-host dispersal probability (e.g. by metabarcoding

approaches searching for symbiont species on atypical host species), as well as on symbiont and host traits favoring host-switching. Theoretical modeling approaches would then allow exploring the consequences of the intensity of different factors (transmission potential, settlement probability) on the relevance of host-shift speciation, cospeciation, and other macro- evolutionary processes for symbiont diversification.

We can also gain insight by comparing feather mites with other permanent ectosymbionts with a similar lifestyle such as feather lice (Jovani, 2003), where a similar diversity of cophylogenetic patterns has been found: in some groups cospeciation is more common (e.g. Swiftlet lice, Clayton et al., 2002) while in others there is extensive host-shift speciation (e.g. *Brueelia* lice, Johnson et al., 2002). Interestingly, passerine feather lice (from the genus *Brueelia*) also show evidence for repeated host-switches, as we have found here. However, feather mites do not use phoresis as *Brueelia* feather lice do (Johnson et al., 2002). Our results may therefore suggest that ecological host-related factors other than high symbiont dispersal potential via phoresis may be more important in explaining host-switching in ectosymbionts.

There are several possible and non-exclusive explanations about why *Proctophyllodes* and *Trouessartia* mites exhibit pronounced host- switching. First, their passerine host species have strongly overlapping distributions (Svensson et al., 2010), with frequent opportunities for interspecific (and intraspecific) contacts during dust bathing, aggressions, feeding in flocks or at shared feeding places, which might favor feather mite dispersal (Dubinin, 1951; Herrera, 1979; Zamora, 1990; Jovani and Blanco, 2000; Proctor, 2003; Dabert et al., 2015). Another important factor may be that some host species included in this study are hole-nesters, where interspecific competition for nest holes sometimes results in interspecific nest takeovers (Merilä and Wiggins, 1995). As suggested for *Brueelia* feather lice, contacts derived from this competition can trigger host-switching (Johnson et al., 2002; Clayton et al., 2016). Lastly, flight feather microstructure is very similar among pas- serines (in contrast to that in non-passerine orders, Pap et al., 2015), thus presumably allowing feather mites to colonize new host species after a fortuitous dispersal event, while the lack of further ongoing gene flow may promote local adaptation and eventually speciation, completing the host-shift speciation (Agosta and Klemens, 2008; Johnson et al., 2012). In contrast, the chemical composition of the preen gland oil secretions (one of the candidate

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food resources of feather mites; Proctor, 2003) is known to be highly variable among bird species (Haribal et al., 2009; Whittaker et al., 2010). Therefore, our results may indicate that feather mites are not highly specialized to the specific chemical composition of their host preen oil.

Our findings also represent a remarkable contribution to the phylogenetics of feather mites, in particular for the understanding of the intra-generic relationship of the conflictive genera *Proctophyllodes* and *Trouessartia* (Knowles and Klimov, 2011). Our study is the first applying mitochondrial genome-based phylogenies for the study of feather mites. This mitochondrial marker is also likely to be useful for this group because is less sensitive to incomplete lineage sorting, that may bias phylogenetic results of groups with high speciation rates, such as the feather mites here studied (Moore, 1995; Dabert et al., 2010; Knowles and Klimov, 2011). In addition, potential problems for phylogenetic inference based on mitochondrial markers, such as introgression of mitochondrial lineages and sex-biased gene flow are unlikely to be affecting feather mites' phylogenetic reconstructions. This is because more than one species of the same feather mite genus almost never coexist on the same host (i.e. because of competitive exclusion, Pérez and Atyeo, 1984; Choe and Kim, 1989; Fernández-González et al., 2015; Stefan et al., 2015; Doña et al., 2016), and there is no evidence of hybridization nor sex-biased gene flow among feather mite species. In addition, we know from other symbiont groups that host colonizations (i.e. host-switches) are generally done to a host free of symbionts (e.g. Torchin et al., 2003), thus precluding hybridization as well as sex-biased gene flow. Therefore, in spite of we have inferred the mitochondrial trees, our trees are largely in agreement with previous phylogenetic studies based on morphology and nuclear markers, and thus it is unlikely that they significantly differ from the species tree. In particular, our results for *Proctophyllodes* are highly congruent and revealed the same clade relationships than those found by Knowles and Klimov (2011), and also agree with a recent independent phylogeny (based on six nuclear genes plus COI, covering a total of 34 species from those here studied, Klimov et al., 2017b). These clades are also in agreement with morphological taxonomy (Mironov, 2012). For *Trouessartia* species, the intra-generic relationships within the genus are rather unclear and have been never seriously analyzed. Nonetheless, our tree is largely in agreement (and improves the overall support) of the single extant Bayesian tree based on COI sequences from Doña et al. (2015). Overall, our results

concur with previous supporting mitochondrial genomes as powerful phylogenetic markers for shallow phylogenetic reconstructions (e.g. Thalmann et al., 2013; Cameron, 2014), and suggests that the mitochondrial genome may be suitable for other cophylogenetic studies on intra-generic relationships of groups with high speciation rates where hybridization is unlikely to occur.

Overall, this study supports host-shift speciation as the main diversification process even for highly specialized and host-specific symbionts. This is in congruence with what it was previously found in other highly specialized symbionts, such as chaetodactylid mites of bees (Klimov et al., 2007) and *Spinturnix* mites of bats (Bruyndonckx et al., 2009), which are also strongly influenced by host-switches. Therefore, highly specialized symbionts are likely to be evolutionarily more dynamic than currently thought. This demands future studies for understanding how host and symbiont-related factors shape host-switches on ecological timescales, and the extent of their global impact on the eco-evolutionary dynamics of highly specialized symbionts. Experimental host-switching approaches, large-scale phylogenies, and extensive field molecular surveys of symbionts (environmental DNA methods, such as DNA metabarcoding) are encouraged.

Chapter 7.

Host specificity, infrequent major host switching and the diversification of highly host-specific symbionts: The case of vane-dwelling feather mites

Abstract

Highly host-specific symbionts are very rarely found except with their typical host species. Although switches to new hosts are rare and difficult to detect, a switch to a host phylogenetically distant from the original one (a 'major host switch') could allow diversification of the symbionts onto the new host lineage. The consequences of such major host switches on the diversification of highly host-specific symbionts of animals have rarely been explored. Here, we examine the host specificity of vane-dwelling feather mites, a group that shows strong specificity, together with their host-switching dynamics and the consequences of major host switches for their diversification. Using the largest published dataset of feather mite–bird associations, we analysed raw, phylogenetic and geographical host specificity of feather mites. We studied host-switching dynam- ics by describing the sharing by feather mites of bird species with different phylogenetic distances. For three of the most species-rich feather mite families, we quantified the consequences of major host switches for feather mite diversification. Most feather mite species (84%) inhabit one to three very closely related host species. Assemblages of feather mites on birds do not show a geographical signature, but rather show strong host-driven structuring. The probability that a mite species occurs on two host species decays sharply with host phylogenetic distance, with only one instance of a feather mite species occupying distantly related hosts from different orders. However, results suggest that despite the strong host specificity, a few major host switches triggered the origin of 21% of the species and 38% of the genera of the mite families studied. We show that feather mites are highly host-specific symbionts, whose assemb- lages do not show geographical structure, even at a continental scale. We conclude that major host switches are very rare events with strong macroevolutionary consequences for feather mite diversity.

Introduction

Rare, sporadic events are difficult to study owing to small sample size and difficulty in prediction and detection, but they can shape major patterns in ecology and evolution. For example, the arrival of new insect species on oceanic islands, or the long-distance dispersal of plant seeds, can result in major consequences for evolutionary radiations and global biogeography (Darwin, 1859; Nathan, 2006). Host switching (the successful colonization of a new host species by a symbiont species) falls into this category; switches are rare and difficult to detect, but because successful switches can isolate symbiont populations in new ecological contexts, they have a high potential to drive diversification of symbionts (Calatayud et al., 2016; Clayton et al. 2016; de Vienne et al., 2013; Hoberg & Brooks, 2008, 2015; Johnson et al. 2011; Martinů et al., 2015; Nyman, 2010; Poulin, 2011; Ricklefs et al., 2014). Although symbionts constitute much of Earth's biodiversity (Dobson et al. 2008; Larsen et al. 2017; Morand, 2015), we still lack a solid understanding of host-switching dynamics and how it shapes symbiont evolution.

The probability of host switching is not the same for all groups of symbionts, being more likely in generalists that move among hosts using horizontal dispersal than in highly specialized symbionts that are primarily transmitted vertically from parents to offspring (Clayton et al., 2016). Nevertheless, one general pattern shared among symbionts is that successful switches occur mainly between phylogenetically closely related hosts ('clade-limited switches'; Braga et al. 2015; Krasnov et al. 2004; Krasnov et al., 2010; Poulin, 1992, 2010, 2011; Poulin et al. 2011). However, on rare occasions, symbionts may establish on phylogenetically distant hosts ('major host switches'; Calatayud et al., 2016; Johnson et al. 2011; Martinů et al., 2015; Nyman, 2010; Poulin, 2011; Torchin et al. 2003). Subsequent speciation on the new host can initiate a co-evolutionary radiation (Clayton et al., 2016). Examples include radiation of the monogenean genus *Gyrodactylus* on distantly related fish families (Zietara & Lumme, 2002), or Goniodidae feather lice switching from galliform to columbiform birds and back again to one lineage of galliforms (Johnson et al., 2011). With these exceptions, the consequences of such major host switches on diversification of animal-associated symbionts remain almost unstudied (Johnson et al., 2011).

Astigmatan feather mites (Acariformes: Astigmata: Analgoidea and Pterolichoidea; hereafter, feather mites) are the most abundant and diverse ectosymbionts of birds (Dabert

& Mironov, 1999; Diaz-Real et al. 2014; Doña et al. 2016; Gaud & Atyeo, 1996; Proctor, 2003; Proctor & Owens, 2000). Among them, vane-dwelling feather mites have a relatively simple life cycle (egg, larva, protonymph, tritonymph, adult) that is completed entirely on the flight feathers of their host. Feather mites cannot survive off the host (Dubinin, 1951; Proctor, 2003) and have very restricted transmission capabilities because they lack a specific stage for transmission found in many free-living astigmatan mites (a morphologically modified deutonymph also referred to as a hypopus), and other stages do not engage in phoretic behaviour (i.e., they do not attach to larger and more mobile symbionts for transmission, in contrast to many feather lice that hitch rides on hippoboscid flies). Hence, they are mainly transmitted vertically from parents to offspring (Doña et al., 2017; Jovani et al. 2001; Proctor, 2003). Feather mite species are often highly host specific and restricted to a single bird species, usually occurring in specific wing areas and even in particular sections within feathers (Choe & Kim, 1989; Fernández-González et al., 2015; Jovani & Serrano, 2001, 2004; Mironov, 1999; Proctor, 2003; Pérez & Atyeo, 1984; Stefan et al., 2015; Walter & Proctor, 2013).

Given this biology, it is surprising that a recent cophylogenetic study between two genera of feather mites and European passerine birds (Doña et al., 2017) showed that host switching, rather than host–parasite cospeciation (i.e., symbiont speciation after host speciation), may be the main driver of diversification. In fact, cophylogenetic reconstructions showed that speciation after host switching was more prevalent by far than cospeciation in the passerine feather mites studied. In addition to these switches among closely related passerine hosts, major switches between host orders have also been reported in feather mites (Dabert, 2004; Gaud, 1992; Gaud & Atyeo, 1996; Hernandes et al. 2014). This is the case for the proctophyllodid feather mites of hummingbirds (Apodiformes: Trochilidae), whose ancestor switched from passeriform hosts (Knowles & Klimov, 2011; Mironov, 2009).

As part of a long-term study of the degree of host specialization of feather mites, we built the largest published dataset of bird–feather mite associations: 1,876 mite species inhabiting 2,144 bird species (Doña et al., 2016). Here, we used this dataset to study raw, phylogenetic and geographical host specificity (following Poulin et al., 2011). Then, we examined host-switching dynamics by describing the sharing of feather mites among bird species with different phylogenetic distances. Finally, for three of the most species-rich

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feather mite families (a total of 823 mite species from the Proctophyllodidae, Pteronyssidae and Trouessartiidae), we quantified the relevance of major host switches for feather mite diversification.

We expected to find high host specificity given the known biology and ecology of feather mites, such as their low transmission capabilities, which would hamper host switches (see above). We thus predicted that most mite species would inhabit a small number of host species (raw host specificity), which would be phylogenetically more closely related than predicted by chance alone (phylogenetic host specificity), and that most variance in mite assemblages would be explained by host identity rather than by geography (at the level of continents). Major host switches were expected to be very rare, but potentially associated with the evolutionary diversification of feather mites.

Materials and methods

Data

Our global bird–feather mite database (see Doña et al., 2016 for more details) was pruned to analyse only high-quality data, selecting 11,336 bird–mite associations with quality category 2 (i.e., mite species repeatedly reported from the same host bird species), and then keeping only 4,423 unique host–symbiont associations between 1,876 feather mite and 2,170 bird species (Figure 1).

FIGURE 1 Geographical distribution of data on bird–feather mite associations. Countries are coloured according to the number of records on a logarithmic scale. Modified from Doña et al. (2016)
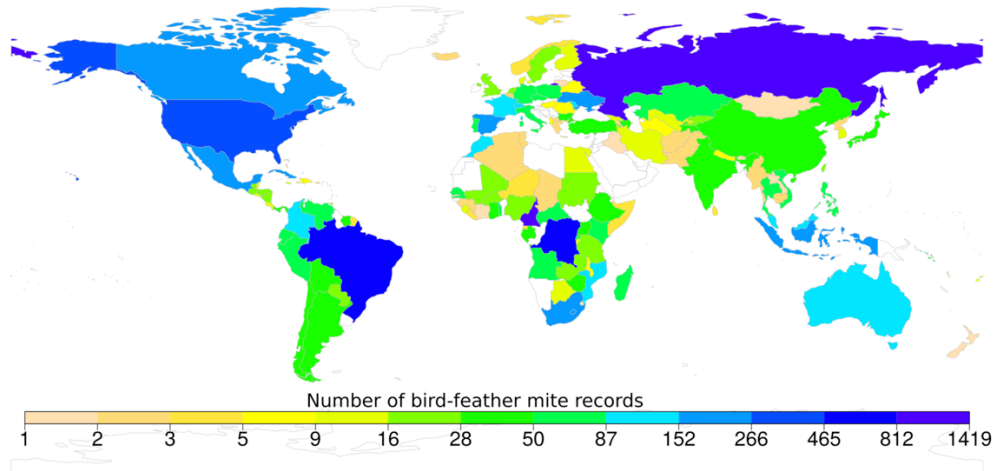
Number of bird-feather mite records

1   2   3   5   9   16   28   50   87   152   266   465   812   1419

Host phylogenetic information was obtained from BirdTree (Jetz et al. 2012; http://birdtree.org). We downloaded 1,000 trees from the Ericson backbone tree, and then summarized them by computing a single 50% majority-rule consensus tree using *SumTree v 4.1.0* in *DendroPy v4.1.0* (Sukumaran & Holder, 2010, 2015), following Rubolini et al. (2015). We found phylogenetic information for 2,134 out of the total 2,170 bird species, which included at least one host for all feather mite species in our study. Avibase information (accessed on March 2016; Lepage et al. 2014) was used to match avian taxonomy in Doña et al. (2016) with that of Jetz et al. 2012 (Supporting Information Table S1). All analyses were carried out in the *R* environment (*R* Core Team, 2015).

Host range

For each feather mite species, we calculated its host specificity in the basic sense (i.e., the number of recorded hosts or 'host range'; sensu Lymbery, 1989). We also analysed host specificity separately for the four best-sampled feather mite families (Alloptidae, Proctophyllodidae, Pteronyssidae and Trouessartiidae; Supporting Information Figure S1; Doña et al., 2016).

Phylogenetic specificity

Raw host specificity does not account for the phylogenetic relationships among hosts; if that is incorporated, it is termed 'phylogenetic specificity' (Poulin et al., 2011). We calculated the standardized effect size of Faith's phylogenetic diversity (PD) of hosts exploited by feather mites (of those mite species with more than one host) using the *R* function *ses.pd* from *Picante* (Kembel et al., 2010). The standardized effect size of phylogenetic diversity (SES.PD) is the difference between the observed phylogenetic diversity between hosts of each mite species and the mean phylogenetic diversity obtained with the same number of hosts generated using a random choice of host species from the tree (null data), divided by the standard deviation of phylogenetic diversities in the null data. We performed 999 runs, 1,000 iterations and, as recommended by Poulin et al. (2011), we used 'taxa.labels' to shuffle taxon names in the host phylogenetic tree to act as a null model. Positive SES.PD indicates low

phylogenetic specificity (i.e., greater host phylogenetic diversity than expected by chance), whereas negative SES.PD indicates high phylogenetic specificity (Poulin et al., 2011; Procheş et al. 2006; Webb, 2000). The same rationale was applied to the net effect size (NES), calculated as the observed PD minus the null average of simulated PD.

Geographical specificity

We also analysed the geographical aspect of host specificity (Poulin et al., 2011). Birds are potentially exposed to mite species from sympatric bird species, which could result in feather mite assemblages being (at least in part) geography specific rather than host specific (Poulin & Keeney, 2008; Poulin et al., 2011).

For the analyses detailed below, we chose the mite assemblage per bird species per continent as our analytical scale unit because this is the scale allowing higher completeness in our dataset (Doña et al., 2016). Additionally, we repeated the analyses while taking into account the pathways used by many bird species during migration (mainly south–north) between continents (e.g., between Europe and Africa), and thus that the same bird species might have been studied for feather mites in two continents. These mite samples from different continents may overestimate the host species effect relative to the geographical effect. Therefore, we re-ran all the analyses while collapsing those continents connected by bird migration, which we call hereafter 'supercontinents': America (North, Central and South America), EurAfrica (Europe plus Africa) and AsiaOce (Asia plus Oceania). Only hosts sampled in more than one continent or supercontinent were included (Antarctica was not considered in any analysis because no record fulfilled this requirement). Our final matrix for geographical specificity included 804 host–continent associations comprising 342 bird and 1,776 feather mite species, and 487 host–supercontinent associations comprising 227 bird and 1,773 feather mite species. Lastly, to minimize the potential effect of sampling heterogeneity across hosts and geography, we re-ran all analyses using only birds with at least three mite species sampled in each geographical area (continental scale: 234 bird–continent associations, 99 bird species and 272 mite species; supercontinental scale: 137 bird–supercontinent associations, 64 bird species and 182 mite species).

We studied the variance in host-based mite assemblages (i.e., all feather mite species inhabiting a bird species) among bird species using a permutational multivariate analysis of

variance on Jaccard distance matrices (PERMANOVA; *adonis* function from the *VEGAN v2.4.1 R* package; Oksanen et al., 2016). Additionally, because Jaccard dissimilarity is a measure affected both by species replacement and by nestedness (Baselga, 2010, 2012), we also conducted our PERMANOVAs separately on dissimilarity matrices from each of these components (e.g., Gómez-Rodríguez et al., 2015). The analysis of the species replacement component measures whether the dissimilarity of assemblages is attributable to the effect of replacement (i.e., dissimilarity because of different symbiont species between host species, after removing the effect of nestedness), whereas the analysis of the nested-ness component measures the dissimilarity of assemblages attributable purely to the effect of nestedness (i.e., when hosts with fewer symbiont species have a subset of those symbiont species inhabiting hosts with more symbiont species; Baselga, 2010, 2012). Dissimilarity matrices were obtained using the *beta.pair* function from the *betapart v1.4- 1* package (Baselga & Orme, 2012). For PERMANOVAs, any negative variance components were set to zero (Graham & Edwards, 2001), and the null hypothesis was that the centroid does not differ between host species and geographical scale (Anderson & Walsh, 2013). This test is known to be sensitive to the usual data dispersion (Anderson, 2001), and we tested this with the multivariate homogeneity PERMDISP2 procedure (Anderson, 2006; betadisper function from VEGAN; Ander- son & Walsh, 2013) with 999 permutations. Indeed, we found different levels of dispersion between host species (continent treatment: $F= 2.220$, $p= .001$; supercontinent treatment: $F= 2.956$, $p= .001$) and between continents ($F= 4.942$, $p= .001$) but not between supercontinents ($F= 0.878$, $p= .385$). Following previous approaches to circumvent this statistical issue (e.g., Steinert et al., 2016), we carried out a hierarchical cluster analysis with the *hclust* function (*STATS* package) using the 'complete' agglomeration method to visualize the clustering of mite communities across bird species and geographical scales.

Potential data biases

First, the identification of mite species in our dataset is mostly based on mite morphology (see Doña et al., 2016 for details). Even though feather mite taxonomy is relatively robust (i.e., it holds up well when compared with molecular-based taxonomy; e.g., Doña et al., 2015) and our dataset is comprehensively curated (Doña et al., 2016), a moderate number of cryptic species may occur in our dataset. Their presence would reinforce our conclusions

because accounting for the existence of cryptic species would increase even more the already very high host specificity reported here (see below).

Second, heterogeneity in sampling effort in our database could overestimate the host specificity of poorly sampled species (for more details, see Doña et al., 2016). However, we did not find variation either when comparing host-specificity patterns among the four best-sampled mite families or between them and the whole dataset (Figure 2; Supporting Information Figure S1). Additionally, we found a strong relationship between the number of hosts known for a mite species and the phylogenetic host specificity ($p<.001$, $R^2_{adj}= 0.77$; Supporting Information Figure S2), suggesting that although increasing sampling effort might potentially decrease raw host specificity (i.e., increase the number of reported hosts), the degree of phylogenetic host specificity will probably be even higher (i.e., in spite of increasing the chances of finding a distantly related host). Thus, our analyses strongly suggest that our conclusions are robust.

Ecological patterns in host switching

We compiled all pairs of bird species connected by each mite species (e.g., if a mite species had four host species, six bird pairs were recorded). Then, we calculated the phylogenetic distance between each bird species pair ($n= 11,056$), and finally, we calculated the proportion of bird pairs (y axis of Figure 4) falling within 10 phylogenetic distance bins ranging from within genus to between orders (x axis of Figure 4). Thus, we were calculating the probability that a bird pair sharing a feather mite has a given phylogenetic distance (i.e., Figure 4 y-axis probabilities sum 1). Phylogenetic distance was measured as the pairwise difference in total branch lengths of species on the bird phylogenetic tree with the function *cophenetic.phylo* from the *APE v4.0 R* package (Paradis et al., 2004).

We used this analysis as a way of studying host-switching dynamics; however, the fact that two closely related bird species (i.e., two species in the first bin of the x axis of Figure 4) share a feather mite species could also be because the mite did not speciate after bird speciation (i.e., failure to speciate; Johnson et al. 2003). This alternative scenario is theoretically possible and is the basis for Manter's first rule (that parasites evolve more slowly than their hosts; Brooks & McLennan, 1991); however, at the genetic level this process would require more gene flow among symbionts than among their hosts (Johnson et al., 2003), and

this is an unlikely scenario for feather mites given their transmission limitations (see above, but also see Dabert et al., 2015). Also, some studies have shown that mites have higher substitution rates than their hosts (Stefka et al., 2011). Overall, the prevalence of failure-to-speciate events is expected to be low and, in any case, would result in only slight overestimation of the first bin of Figure 4.

Evolutionary consequences of host switching

The focal feather mite families Proctophyllodidae, Pteronyssidae and Trouessartiidae make up a large proportion of species in the dataset (823 out of 1876, 44%). Taxon richness, host biogeography and double calibrated cophylogenetic reconstructions (for Proctophyllodidae) support the origin of Proctophyllodidae and Trouessartiidae on passeriform hosts (Gaud & Atyeo, 1982, 1996; Klimov et al., 2017; Mironov, 2009, 2016). This is supported by the molecular validation of the monophyly of the pterodectinae lineage of proctophyllodids on Apodiformes as a consequence of a major host switch from Passeriformes (Knowles & Klimov, 2011). The primary host order of origin of Pteronyssidae is more problematical, as it may have originated either on the Passeriformes or on the Piciformes (woodpeckers and relatives; Gaud & Atyeo, 1982, 1996; Mironov, 2009, 2016); therefore, for this family we analysed both possibilities of origin. In addition, molecular studies have found pteronyssid lineages from Passeriformes and Piciformes to be reciprocally monophyletic (Klimov & O'Connor, 2008, 2013), thus strongly suggesting that a single major host switch occurred either from Passeriformes to Piciformes or vice versa. The observation of a particular species of these families found on host taxa other than passerines/piciforms and not on any passerine/piciform hosts (to exclude very recent major host switches; see Results and Discussion) is evidence that speciation occurred as consequence of at least one major host switch (and subsequent diversification by shorter host switches or cospeciation; see Discussion). After pruning the dataset to retain only Proctophyllodidae, Trouessartiidae and Pteronyssidae, the final matrix included 2,010 unique associations between 823 mite and 1,110 bird species, for which we found phylogenetic information for all host species. Next, we calculated the percentage of species and genera whose host relationships cannot be explained in the absence of major host switches by dividing the number of species from each mite family associated with non-passerines by the total number of species of each mite

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family. For Pteronyssidae, we also calculated these percentages when considering the alternative scenario of origin on piciform hosts. The figure of host–mite associations on the bird phylogenetic tree (Figure 5) was generated with the *trait.plot* function of *Diversitree v0.9-9 R* package (FitzJohn, 2012).

Results

High raw and phylogenetic host specificity

Most feather mite species (84%) have been recorded from one (57%), two (18%) or three (9%) bird species (Figure 2). Those found on more than one host species very rarely occupy hosts from different genera, and even less commonly from different families or orders (Figure 2). In fact, only a single mite species is regularly known from bird species of two bird orders: *Proctophyllodes anthi* Vitzthum on Passeriformes (*Lullula arborea* L., *Motacilla flava* L. and seven species of *Anthus*) and one species of Piciformes (*Jynx torquilla* L.). Consistent results were found for the four best-sampled mite families separately (Alloptidae, Proctophyllodidae, Pteronyssidae and Trouessartiidae; Supporting Information Figure S1). Moreover, the vast majority (99%) of mite species with more than one host ($n= 809$) showed values of phylogenetic host specificity (SES.PD and NES.PD) lower than zero, thus revealing high host phylogenetic clustering (i.e., high host phylogenetic specificity; Supporting Information Table S2).

FIGURE 2 Number of feather mite species per bird taxon. Values on the y axis represent the number of mite species, and values on the x axis represent the number of bird taxa.
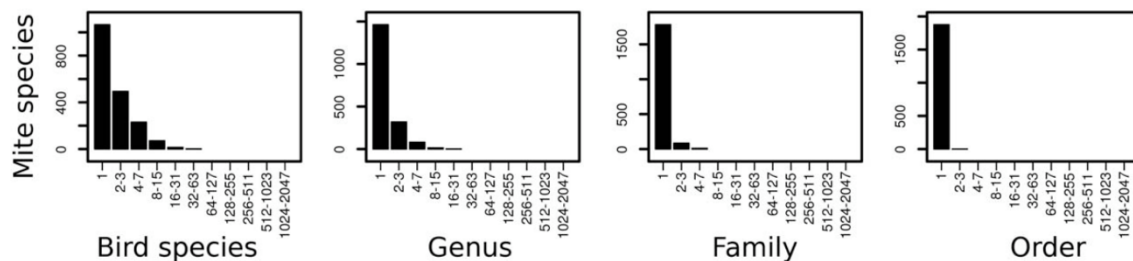
Host taxon versus geography

The identity of the bird species, but not geography, was the main factor determining structure of feather mite assemblages; birds of a given species usually have similar feather mite assemblages in different continents. Most variance of feather mite species composition was explained by bird species identity when comparing their feather mite assemblages between continents (PERMANOVA, 78% variance, $F=$ 4.843, $p=$ .001) and supercontinents (PERMANOVA, 80% variance, $F=$ 4.679, $p=$ .001). In particular, differences in patterns of variation in mite assemblages between bird species were significantly related to mite species turnover between host species (PERMANOVA, continent scale: 87% variance, $F=$ 9.207, $p=$ .001; supercontinent scale: 90%, $F=$ 10.950, $p=$ .001) and not to the nestedness component (PERMANOVA, continent scale: 0% variance, $F=$ 21.331, $p=$ .968; supercontinent scale: 0%, $F=$ 21.123, $p=$ .930). On the contrary, we found no effect of geography as a predictor of the mite assemblages composition (PERMANOVA, continent scale: 0.3% variance, $F=$ 1.349, $p=$ .001; supercontinent scale: 0.24%, $F=$ 1.571, $p=$ .001), on neither turnover (PERMANOVA, continent scale: 0.19% variance, $F=$ 1.349, $p=$ .001; supercontinent scale: 0.11%, $F=$ 1.511, $p=$ .001) nor nestedness components (PERMANOVA, continent scale: 67% variance, $F=$ 1.291, $p=$ .440; supercontinent scale: 44%, $F=$ 1.64, $p=$ .448), thus different bird species sharing a continent or supercontinent did not have geographically specific mite assemblages. Restricting PERMANOVAs to high-quality data (see Materials and methods) confirmed our results of the relevance of bird species (PERMANOVA, continent scale: 82% variance, $F=$ 6.259, $p=$ .001; supercontinent scale: 85%, $F=$ 6.629, $p=$ .001) against geography (PERMANOVA, continent scale: 0.7%, $F=$ 1.120, $p=$ .08; supercontinent scale: 0.5%, $F=$ 1.248, $p=$ .06). Also, similar results were obtained for both predictors for species turnover and nestedness components. So again, bird species contributed significantly to the species turnover (PERMANOVA, continent scale: 88% variance, $F=$ 10.035, $p=$ .001; supercontinent scale: 92%, $F=$ 13.933, $p=$ .001) but not to the nestedness component (PERMANOVA, continent scale: 0% variance, $F=$ 21.317, $p=$ .901; supercontinent scale: 0%, $F=$ 21.104, $p=$ .915). And again, geography contributed significantly to neither species turnover (PERMANOVA, continent scale: 0.45% variance, $F=$ 1.027, $p=$ .376; supercontinent scale: 0.12%, $F=$ 0.579, $p=$ 1) nor nestedness components (PERMANOVA, continent scale: 83% variance, $F=$ 0.898, $p=$ .409; supercontinent scale:

64%, *F*= 1.324, *p*= .410). Moreover, dendrograms from a hierarchical cluster analysis based on Jaccard dissimilarity matrices supported a strong clustering pattern explained by host species rather than geography (Figure 3; Supporting Information Figure S3).

FIGURE 3 Dendrogram representing feather mite community similarity (Jaccard distances) for populations of the same bird species in different supercontinents. All bird species with at least two supercontinents and with records of at least two feather mite species in each supercontinent are displayed. Each text colour represents a different bird species. Each circle colour represents a different supercontinent: America (orange), AsiaOce (blue) and EurAfrica (green)
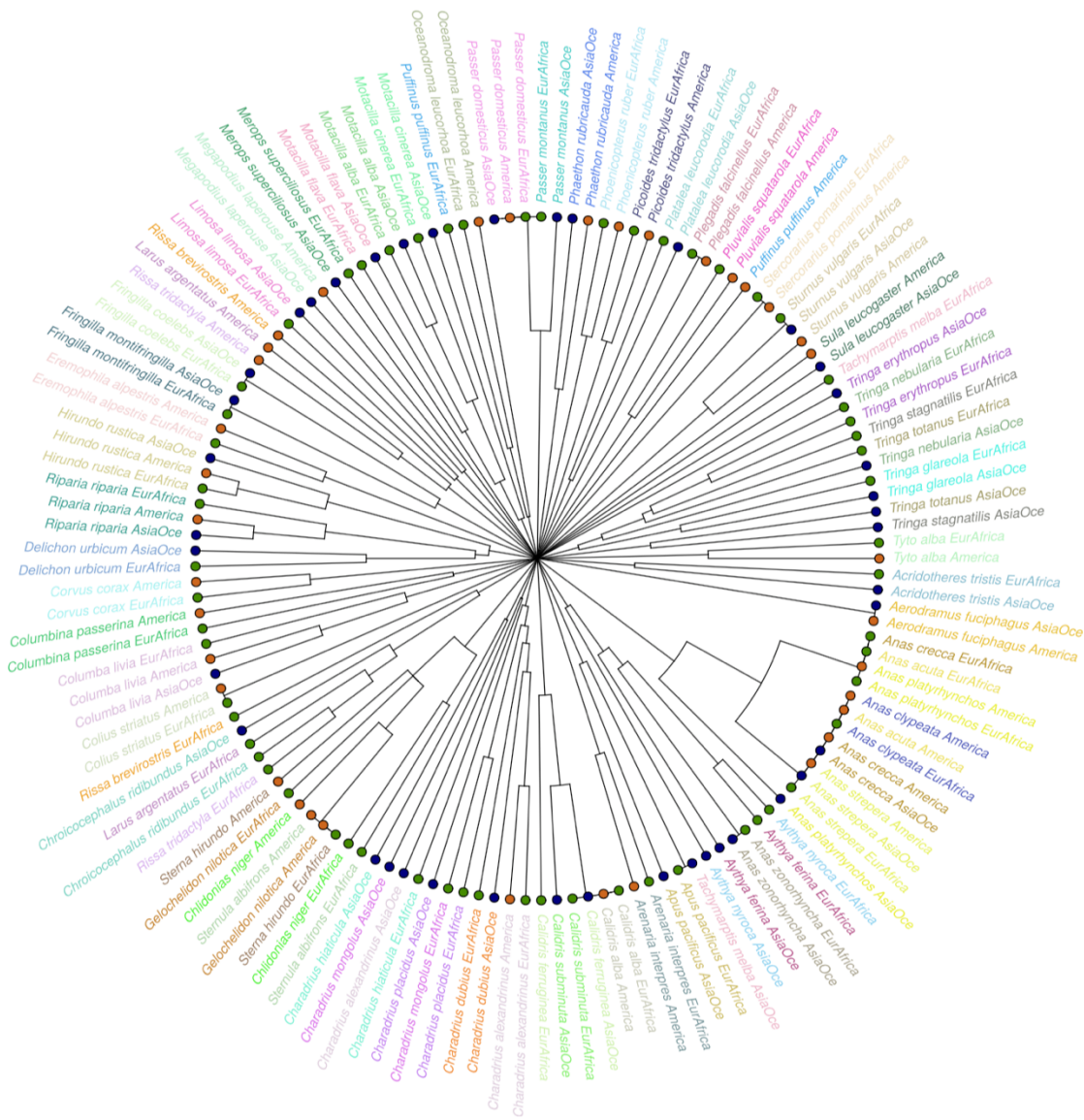
Ecological patterns in host switching and consequences for feather mite diversification

The probability that a pair of bird species shares a feather mite species decays sharply with bird phylogenetic distance (Figure 4).

FIGURE 4 Probability that a bird pair with a mite in common have a particular phylogenetic distance. Close congeners have low distances, and species from different orders have the highest distance. As an example, dashed lines at the x axis mark the phylogenetic distances between primary [tree pipit (Anthus trivialis)] and secondary hosts inhabited by the mite Proctophyllodes anthi (see main text for discussion). Secondary hosts, from left to the right: olive-backed pipit (Anthus hodgsoni Richmond), western yellow wagtail (Motacilla flava), woodlark (Lullula arborea) and Eurasian wryneck (Jynx torquilla). Error bars represent confidence intervals (α 5 0.05)

Phylogenetic distance between bird species sharing mite species
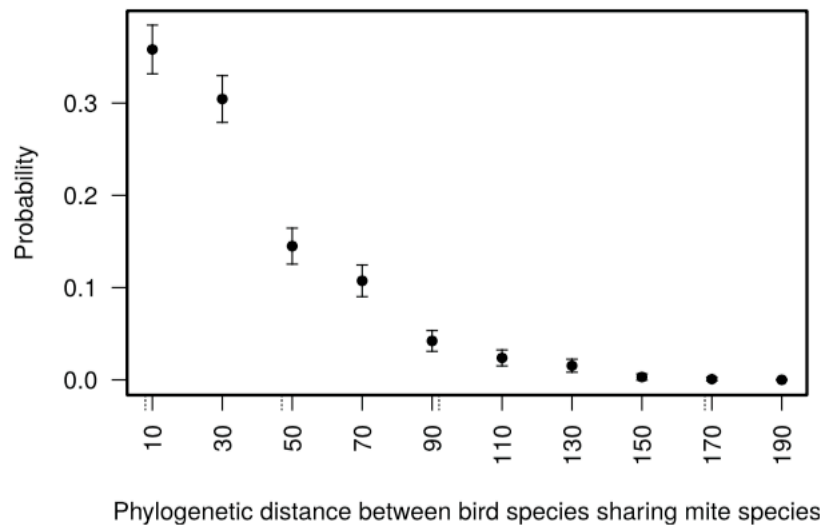
Nevertheless, infrequent major host switches (i.e., among bird orders such as that of P. anthi; see Results' subsection 3.1) have strongly shaped the diversification of feather mites. Specifically, 13% of species and 24% of the genera of the Proctophyllodidae in our analysis evolved after at least one major host switch from Passeriformes to Apodiformes (Figure 5) and subsequent radiation with further (clade-limited) host switches and/or other macroecological events, such as cospeciation. The proportion of 'post-switch' origins is even more striking in the other two mite families: 15% of species and 57% of genera in the Trouessartiidae arose after a major host switch, 43% of species and 54% of genera in Pteronyssidae for the 'Passeriformes origin' scenario, and 53% of species and 45% of pteronyssid genera for the 'Piciformes origin' scenario (Figure 5). Overall, major host switches

appear to have triggered the diversification of 21% of the species and 38% of the genera of these three families (Figure 5).

FIGURE 5 Bird phylogenetic tree depicting the associations of mite genera of (a) Proctophyllodidae, (b) Trouessartiidae and (c) Pteronyssidae. Non-passerine hosts are those placed below the most external black semi-ring, and passerine hosts are those placed below the grey semi-ring. Blue: feather mite genera inhabiting only passerine hosts. Orange: mite genera inhabiting both passerine and non- passerine hosts. Green: mite genera inhabiting only non-passerine hosts. Numbers above the trees depict the total number of species and genera on passerine (values to the right) and non-passerine (values to the left) hosts for each mite family. Dotted arrows indicate direction of major host switches, which for Proctophyllodidae and Trouessartiidae is from passerine to non-passerine hosts. The direction of switch for Pteronyssidae is uncertain (see main text for details)
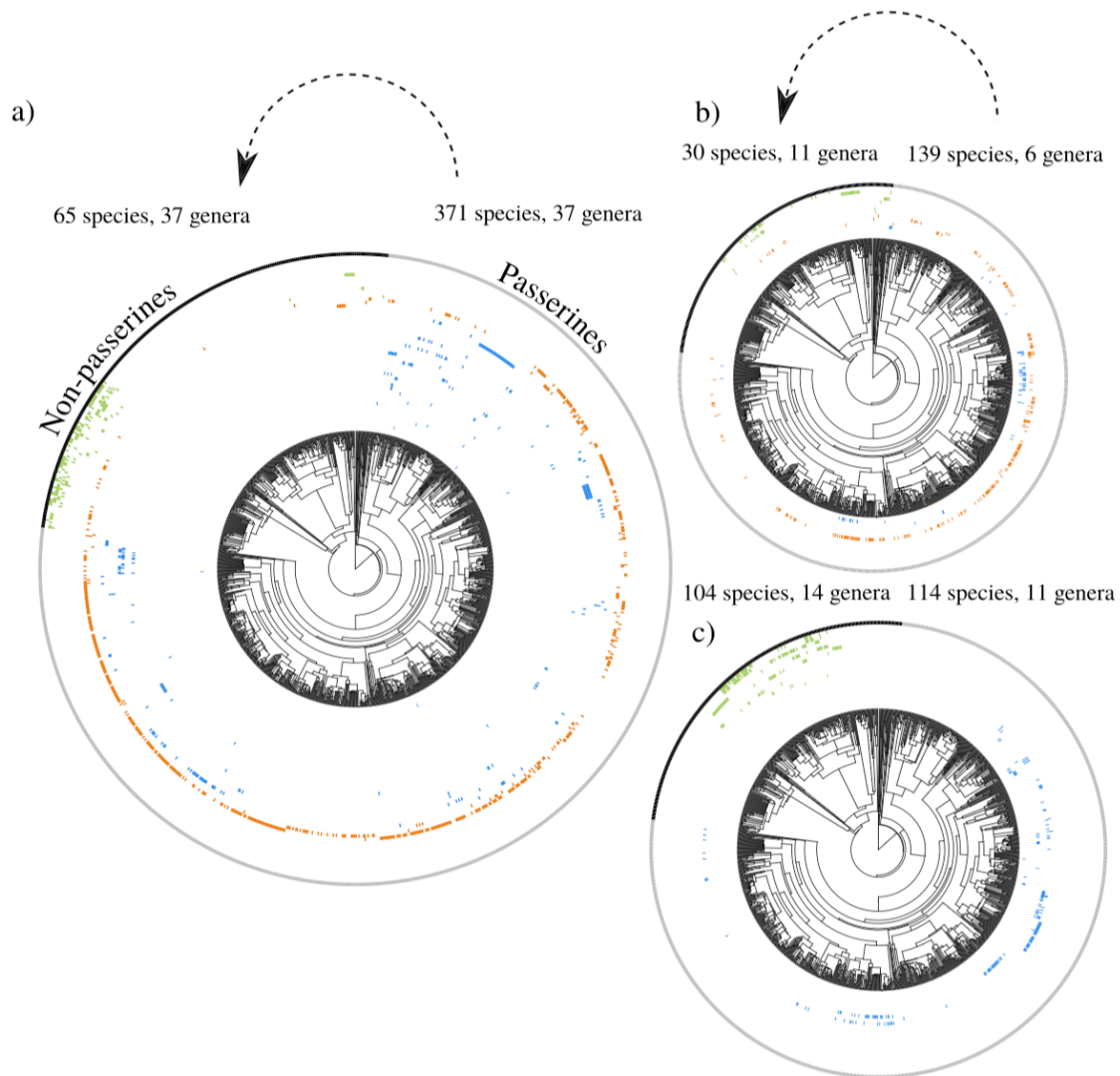
Discussion

Our results confirm that feather mites are highly specialized symbionts, with most mite species inhabiting one or a very few strongly phylogenetically clustered host species. Moreover, feather mite assemblages do not show a geographical signature, but rather strong host-driven structuring across continents (i.e., bird species host the same feather mites in different continents). In addition, these differences in mite assemblages were mainly explained by mite species turnover between bird host species (i.e., feather mite assemblages strongly differ between bird species). Altogether, our results depict feather mites as strongly limited by transmission opportunities or highly constrained by finding similar ecological conditions on the new host, as suggested by mite species inhabiting hosts with a close phylogenetic relationship (see also Agosta & Klemens, 2008; Agosta et al, 2010; Araujo et al., 2015; Combes, 1991; Khokhlova et al., 2012; Poulin, 2011). Interestingly, the opposite pattern has been reported for other symbionts with much higher dispersal capabilities than feather mites, such as rodent fleas (Krasnov et al., 2010), tapeworm fish parasites (Bouzid et al., 2008) and, recently, for spider mite symbionts of plants (Calatayud et al., 2016). Indeed, these contrasting results within mites support that major host switches are particularly unexpected in highly specialized symbionts.

However, although major switches are a rare occurrence, some feather mites have apparently been able to thrive on phylogenetically distant bird species (Figures 2 and 3). Specifically, 95 mite species from our database (5% of all species) are known currently to occupy birds from different families, and there is a single known case of an extant mite species (0.05%) having hosts in two orders (Figure 2). This cannot be explained by the mite failing to speciate after host speciation because of the long time since the bird lineages separated (62 Ma according to Prum et al., 2015), and thus can be explained only by recent major host switching. This is wonderfully exemplified by *P. anthi*, for which the primary hosts are pipits (Passeriformes: *Anthus* spp.), but which also inhabits the Eurasian wryneck (Piciformes: *Jynx torquilla*; Figure 4). This association has been confirmed by several independent taxonomic studies all along the host breeding range from the Iberian peninsula to Japan (Doña et al., 2016). Our molecular study (Doña et al., 2015) on *P. anthi* collected from *Anthus* and *Jynx* hosts found very little genetic differentiation (0.6% of raw genetic distance in COI mitochondrial gene), much less than that necessary to consider them

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different species and much lower than that found for some other named *Proctophyllodes* species living on closely related bird species [e.g., *P. musicus* Vitzthum, 10% of raw genetic distance in COI mitochondrial gene between mites sampled from the common blackbird (*Turdus merula* L.) and song thrush (*Turdus philomelos* Brehm)], which turned out to be cryptic mite species.

Although these major host switches are rare, they may be relevant at an evolutionary time scale. In fact, our study revealed that they have probably triggered the origin of 21% of the species and 38% of the genera of the Proctophyllodidae, Pteronyssidae and Trouessartiidae. In addition, many non-passerine hosts have never been studied for feather mites (Doña et al., 2016), so the real percentage of mite species from these three families on other non-passerines is likely to be underestimated. Overall, occasional major host switches are known to provide novel opportunities for diversification (e.g., Johnson et al., 2011); also, in quantitative terms, our study highlights that they can be uncommon ecological processes with relevant macroevolutionary consequences for the diversification of highly specialized symbionts. Although this was unexpected because of the apparent low potential for horizontal transmission of feather mites, it turns out that this may be the explanation for the macroevolutionary importance of these events; although major host switches are rare, they may lead to diversification because gene flow with the donor population is unlikely and because of the new, probably different ecological conditions found on the new, phylogenetically distant host (Agosta & Klemens, 2008; Agosta et al., 2010; Forbes et al., 2017; Janz, 2011; Nyman, 2010; Zietara & Lumme, 2002). Note that once a mite species successfully colonizes a phylogenetically distant host species (as for *P. anthi*) and probably becomes adapted to the new host species, new host switches would be more likely towards host species closely related to the new host (i.e., clade-limited switches; see Figure 4), and host-shift speciation may take place. This, mainly in combination with cospeciation, may explain subsequent diversification after a major host switch.

These major shifts may be facilitated by bird behaviour promoting contact between different species, such as predation, flock sharing, sand-bath sharing, using allospecific feathers for nest building, and sharing (or stealing) nest cavities. However, once on the new, phylogenetically distant host, the feather mite species must be able to establish a persistent population, and this is likely to depend on host characteristics and mite pre-adaptations to

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them. This is nicely exemplified by the fact that the few genera occurring both on passerines and non- passerines occur on non-passerine species where a different lineage of the same mite family has diversified (Figure 5). For example, representatives of the proctophyllodid genera *Trochilodectes* (which exclusively inhabits hummingbirds, Trochilidae: Apodiformes) and one species of *Proctophyllodes* (which is otherwise almost entirely on passerines) occur on the same trochilid bird host species, the blue-throated hummingbird *Lampornis clemenciae* (Lesson). Interestingly, host switches by Proctophyllodidae and Trouessartidae from their usual passerine hosts are to different non-passerine birds (Figure 5). In Proctophyllodidae, this occurs mostly with *Proctophyllodes* species inhabiting Trochilidae, whereas in Trouessartiidae it occurs with *Trouessartia* species inhabiting Picidae (Figure 5). It is also necessary to add here that these 'recent stragglers' belong to the most species-rich genera of the corresponding mite families. This could mean that species-rich genera include species with a somewhat higher capability of adaptation to new hosts, even from a different bird order, or simply that transmission in these genera is more likely because they are more widespread and thus have higher transmission opportunities.

Our results support the negative relationship between host-switching potential and specialization that has been shown to be relevant in determining the evolution of host specificity in other symbionts, and that occasional major host switches therefore may have an important evolutionary role (Barker, 1991; Johnson et al, 2011; Krasnov et al., 2004; Poulin, 1992, 2011). However, the number of examples is few, and the role of infrequent major host switches on the development of symbiont specialization requires further study. Finally, a recent cophylogenetic study (Doña et al., 2017) carried out at a clade-limited scale for hosts (between passerine species) recovered many more host switches (70–75% of the events for *Proctophyllodes* and *Trouessartia* mites, respectively) than it did cospeciation events. Altogether, this supports diversification of the feather mites as the consequence of a history of both clade-limited and major host switches, with a potentially more minor role of cospeciation. Recent advances in DNA sequencing will allow future cophylogenetic/genomic studies to cover the whole diversity of feather mites, across scales, and to compare the results with other symbiont groups, thus revealing a more complete picture of the diversification history of symbiont species.

Chapter 8.

Chasing host-switches: A DNA metabarcoding study on the eco-evolutionary scenario of host-shift speciation of host-specific symbionts

Abstract

Host-shift speciation is becoming acknowledged as the primary diversification driver of even highly-specialized and host-specific symbionts. However, ecological and microevolutionary processes leading to host-shift speciation are poorly understood. Here we investigate host-switching dynamics in a highly-specialized and host-specific system: feather mites on passerine birds in Spain. Utilizing DNA metabarcoding we successfully sequenced mite mtDNA (COI) separately for 1,130 birds (from 71 bird species) totaling 25,540 individual mites (from 64 species). We identified stragglers as mites found on unusual hosts, according to a recent bird-feather mite associations catalog. Surprisingly, 1,228 (4.8%) individual mites were found as stragglers in 84 (8.1%) samples. Stragglers were widespread across mite and bird species. The abundance of stragglers was on average smaller than that of non-straggler species but in some samples where both showed similar abundances. Stragglers colonized hosts more distantly related than their usual hosts, but with a similar body size. Bird-mite associations were modular, and stragglers appeared more than expected by chance in new hosts belonging to the same module as their usual hosts. Lastly, a 47.4% of stragglers showed genetic signatures of incipient differentiation. Overall, our results show straggling and host-switching as a highly widespread phenomenon even for highly specialized and host-specific symbionts. Thus, rather than a rare ecological-microevolutionary phenomenon with eventual macroevolutionary consequences, these events set the stage for a geographic mosaic of coevolution, where host-symbiont modules would be the arena of highly dynamic coevolutionary and codiversification processes.

Introduction

Host-shift speciation (symbiont speciation after successful colonization of a new host species) is becoming acknowledged as the primary driver for the diversification of symbionts (de Vienne et al., 2013; Clayton et al., 2016; Nylin et al., 2017). This conception makes symbionts active agents of their evolution, with significant applied implications for emerging infectious diseases and the response of highly threatened parasites to climate change (Hoberg & Brooks, 2015; Carlson et al., 2017). However, we are still far from understanding the genesis of host-shift speciation at an ecological and microevolutionary scale, which factors influence it, and how these processes percolate up to the macroevolutionary level.

At a macroevolutionary scale, the signature of host-shift speciation on symbiont diversification has been found in a variety of host-symbiont systems (Ricklefs et al., 2004; de Vienne et al., 2013; Clayton et al., 2016; Bourguignon et al., 2018). Mainly by cophylogenetic studies, we now know that the relevance of host-shift speciation versus other processes such as cospeciation on the diversification of symbionts varies among groups (de Vienne et al., 2013; Clayton et al., 2016 and references therein). Moreover, evidence suggests that some factors, such as symbiont dispersal or parasite ecomorphology, are related to its relevance at a macroevolutionary scale (Sweet et al., 2017; Sweet et al., 2018a). However, the direct observation of ongoing host-switching events is especially challenging because of their presumed scarcity and mostly ephemeral nature, especially in highly specialized and host-specific symbionts known to hardly survive out of their host species.

The study of stragglers may help to understand host-switching. Stragglers are symbionts that are found in a different (new) host from those generally harboring that mite species (i.e., its usual host range). The existence of stragglers has been known for a long time (Kellogg, 1896; Rózsa, 1993; Choudhury et al., 2002) even long before the relevance of host-shift speciation was revealed. Interestingly, though, straggling is the first step for the successful establishment in a new host, and therefore a fundamental process to understand host-switching (Rózsa, 1993; Rivera-Parra et al., 2016). The consideration of host-shift speciation as a marginal ecological dead-end process, even difficult to distinguish from methodological artefacts (e.g., sample contaminations when studying ectosymbionts from hosts in museum specimens, or from hosts stored together before sampling; Ròzsa 1993) has resulted in a historical lack of attention to this process. Current renewed interest in

understanding straggling ecology has shown that most stragglers would likely fail to colonize the new host due to different ecological filters (Rózsa, 1993; Whiteman et al., 2005; Rivera-Parra et al., 2016). However, the study of straggling and host-switching has been profoundly hampered by the lack of appropriate methods to massively study this elusive (but relevant) phenomenon (but see Rivera-Parra et al., 2016). Here we used DNA metabarcoding to tackle this challenge using feather mites as our model system.

Feather mites (Acariformes: Astigmata: Analgoidea and Pterolichoidea) are permanent and highly host-specific ectosymbionts of birds (Dabert & Mironov, 1999; Proctor, 2003), as most species inhabit only one or a few, usually closely related bird species (Doña et al., 2018a). Moreover, mites show specific adaptations to live on their hosts (Dabert & Mironov, 1999; Proctor, 2003): morphological fit to feather microstructure, microsite preferences within host feathers, fine-tuned distributions along bird wings, and behaviors to avoid feathers close to being molted (Jovani & Serrano 2001, 2004; Fernandez-Gonzalez et al., 2015; Stefan et al., 2015). Feather mites lack of specific stages for transmission, and they are not known to disperse by phoresis on parasitic insects associated with birds such as hippoboscid flies and feather lice (Dabert & Mironov, 1999; Jovani et al., 2001; Proctor, 2003; Doña et al., 2017b). Nevertheless, current knowledge suggests that their primary mode of transmission is vertical from parents to offspring in the nest (Doña et al., 2017b). In addition, they likely maintain a mutualistic relationship with birds in which they mostly feed upon fungi and bacteria, and likely on the uropygial gland oil that birds smear on the plumage (Doña et al., 2018b). Thus, such highly-specialized and host-specific symbionts have all ingredients to be closely associated with one or a few host species, diversifying by cospeciation. Interestingly, and contrary to this expectation, recent studies have inferred that host-shift speciation is the primary process driving the evolutionary diversification of feather mites (Doña et al., 2017a; Doña et al., 2018a; Matthews et al., 2018). These results suggest that host-switching, despite its apparent difficulty for feather mites, has left a macroevolutionary fingerprint (e.g., in host-mite cophylogenies) along thousands or millions of years (Doña et al., 2017a; Doña et al., 2018a). Our aim here was to quantify straggling and ongoing host-switching in feather mites, as well as to explore/study/investigate some of their ecological determinants.

We first explored the frequency of straggling using DNA metabarcoding using an extensive dataset. Surprisingly, we found that straggling is highly prevalent, and investigated the ecological filters that can shape this process. Overall, our findings challenge our initial vision of highly host-specific symbiosis as ecologically static systems and highlight the potential relevance of microevolutionary processes in the dynamics of these interactions, which might in turn contribute to the most often studied macroevolutionary patterns.

Materials and methods

Sampling and DNA metabarcoding pipeline

We sampled feather mites during 2010–2015 from live passerine birds captured with mist nets in different localities of Spain (Table S1, Supporting information). We collected all the feather mites found in primary, secondary and tertial feathers from the right wing of each bird using a cotton swab impregnated with ethanol, and preserved mites at -20 °C in tubes with 96% ethanol.

We took particular attention to our sampling protocol to avoid the risk of artificial mite cross-contaminations between bird species (i.e., methodological artifacts rather than true stragglers). A previous study did not find feather mites detached from birds in cloth bags used to transport them from the mist-net to the field station (Fernández-González, 2013). So, for 491 birds (of those from which we succeed sequencing their mites) we used "normal" field procedures. That is, we extracted birds from the mist-net with bare hands, placed them in standard bird ringing cloth bags, and then handled them again with bare hands when sampling their mites using disposable cotton swabs (because of the obvious risk of cross-contamination by reusing them). Moreover, to test whether the prevalence found with this protocol came from cross-contamination when using bare hands or even reused cloth bags, we also applied a "refined" protocol to 639 birds where: 1) we used single-use latex gloves for extracting each bird from the mist net. 2) A single-use paper envelope to carry the bird until the field workstation (some meters away) and store it till processing. 3) A new pair of disposable latex gloves for handling the bird during feather mite sampling using disposable

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cotton swabs. We found that the prevalence of stragglers did not differ between both protocols ("normal": 7.1% (35 out of 491) of samples with stragglers, vs. 7.7% (49 out of 639) in "refined" samples; $\chi 2$= 8.22-31; df = 1; P=1). We also explored potential tagging errors by retrospectively checking whether natural hosts of stragglers were handled up to two birds before or after the focal bird with stragglers (i.e., birds potentially overlapping in time during sampling and thus susceptible of potential tagging interchanges). We found that in 81.0% of the cases, stragglers were found even when a potential tagging error was highly unlikely (note that this does not mean that tagging errors are behind the other 19.0%). Overall, our methodological analyses showed that we could be confident that our results correspond to true (stragglers and non-stragglers) bird-mite associations, and therefore we used samples from both protocols for downstream analyses.

Mites from each sample, representing a bird's mite community (i.e., each field microtube with feather mites from each bird) were counted under the stereomicroscope, and were then analyzed following the DNA metabarcoding pipeline for feather mites described in Vizcaino et al., (in rev.). Briefly, each bird's mite community was placed into one well of a 96-well plate and filled with 96% ethanol, leaving two empty wells for a DNA negative extraction control and a PCR negative control. Then, DNA was isolated using the HotSHOT method (Truett et al., 2000). DNA sequencing libraries were prepared by amplifying a region of the mitochondrial COI gene (Doña et al., 2015a, Doña et al., 2015b, Doña et al., 2018b), and by adding the Illumina-specific sequencing primers, indices, and adaptors in a two-step PCR. Finally, libraries were pooled together and analyzed in a total of eight MiSeq 300PE runs (MiSeq Reagent Kit v3). Obtained reads were quality-checked and quality-trimmed. Then, the Python script (MMIS) was used to automatize: sequence concatenation, OTU picking, and to eliminate mistagging events. Lastly, we also checked if representative sequences contained STOP codons.

Data analyses

Unless otherwise stated, all analyses were carried out in the R environment (R Core Team, 2017). We considered that a bird's mite community contained stragglers when a particular bird-feather mite species association was not reported with confidence (data quality = 2) in the global catalog of bird-feather mite associations. Note that in this database, we reviewed

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all available information from the literature, and S.M. taxonomically curated it carefully (Doña et al., 2016). Samples containing representative sequences unclassified at the species level or containing stragglers were further analyzed by S.M. based on morphological characters of the exoskeletons, thanks to the fact that our DNA extraction protocol preserves this material (Doña et al., 2015a). Also, for bird's mite communities containing stragglers, we registered the proportion of each reproductive stage and sex. Among these molecularly unidentified mites, we found putative new species (which were excluded for downstream analyses because of the impossibility of treating them either as straggle or non-straggle mites). 78.7% of stragglers were also validated morphologically and, from those non-validated, 31.2% only contained nymphal stages in which species-level identification was not possible.

We estimated the intensity (i.e., number of individual mites) of each feather mite species found within each bird's mite community by multiplying the proportion of reads retrieved from each mite species by the total number of feather mites counted in the bird's mite community and then rounding to the nearest integer. We have shown elsewhere that this yields a reasonable estimate of the number of individual mites (Diaz-Real et al., 2015; Vizcaino et al., in rev.).

For each feather mite species, we calculated genetic distances between stragglers and mites inhabiting usual hosts (according to Doña et al., 2016; hereafter non-straggler mites) with the *dist.dna* function ("raw" model) from *APE* (Paradis et al., 2004). First, we aligned representative DNA sequences from stragglers and non-straggler mites of each mite species (only in this analysis we do not use sequences with STOP codons) with *Muscle v3.8.31* using default parameters (maximum number of iterations, 2) (Edgar 2004). Then, alignments were trimmed to discard those columns which contained a significant proportion of gaps using the function *msaTrim* with default parameters (fraction of gaps tolerated at the ends of the alignment, 0.5; fraction of gaps tolerated inside the alignment, 0.9) from microseq v1.2.2 (Snipen & Liland, 2018). Also, we explored the distribution of haplotypes of straggler mites by building haplotype networks with the *haplotype* and *haplonet* (using raw genetic distances) functions from *PEGAS v0.10* (Paradis 2010).

Host phylogenetic information was obtained from BirdTree (Jetz et al., 2012; http://birdtree.org). We downloaded 1,000 trees from the Ericson backbone tree and then

summarized them by computing a single 50% majority-rule consensus tree using *SumTree v 4.1.0* in *DendroPy v4.1.0* (Sukumaran & Holder, 2010, 2015), following Rubolini et al., (2015). We found phylogenetic information for all the bird species studied. Following Doña et al., (2017), Avibase information (accessed on March 2016; Lepage et al., 2014) was used to match avian taxonomy in Doña et al., (2016) with that of Jetz et al., (2012).

To study host phylogenetic specificity of straggler and non-straggler feather mites we estimated (following Doña et al., 2018a) the probability density function of the phylogenetic distances between host species sharing a mite species. To do so, we calculated the phylogenetic distance (as in Doña et al., 2018a) between each bird species pair sharing a mite species and calculated the proportion of bird pairs falling within ten phylogenetic distance bins. Straggler mites and their associations with usual hosts were visualized using the function arcplot from the *arcdiagram v0.1.11* package (Sanchez, 2014).

To understand if host morphology imposes an ecological constraint to straggling we explore the relationships between the phylogenetic distance between usual and unusual hosts (i.e., the host inhabited by a straggler mite) and their differences in body size. Bird body mass is evolutionary conserved, so that closely related species tend to have similar body sizes (Smith & Lyons, 2013). We would expect that if stragglers tend to associate with bird species distantly related from their usual hosts, these species will also differ in their body size compared to the usual hosts. However, if unusual hosts are phylogenetically distant from the usual hosts but with a similar body size, that would suggest that body size imposes a constraint to straggling (Smith & Lyons, 2013; Clayton et al., 2016). For this purpose, we calculated the body mass differences and phylogenetic distances between all pairs of hosts in which a mite species was found (Doña et al., 2016). The phylogenetic distance was measured as the sum of branch lengths from the most recent common ancestor to the two tips (species) of the bird phylogenetic tree with the function cophenetic.phylo from *APE v5.1* (Paradis et al., 2004). We measured body mass distance as the difference between the maximum and the minimum body mass of each pairwise comparison. We obtained body mass information from Dunning (2008).

To further explore the ecology of straggling from a multi-specific (non-pairwise) point of view, we first identified groups of birds and feather mites that tend to associate more among them than with other species in the network of natural associations (i.e., modules),

using the simulated annealing method implemented in the *netcarto* function with default parameters (iteration factor=1; cooling factor = 0.995, bipartite = False) from *rnetcarto v0.2.4* (Guimera & Amaral, 2005a, b; Doulcier & Stouffer, 2015). The adjacency matrix included all the bird species (according to Doña et al., 2016) associated with each mite species from our DNA metabarcoding results. It also included those new host species "colonized" by stragglers and their usual mite species (according to Doña et al., 2016), but not mites classified here as stragglers. Also, to evaluate whether hosts included in each module were more closely related than expected by chance (i.e., phylogenetic signal of hosts included in each module), we calculated the D-statistic using *phylo.d* function from *CAPER v0.5.2* (Fritz & Purvis, 2010). Lastly, we tested whether there is a significant tendency of unusual hosts of straggler mites to be grouped within the same module as their usual hosts. To do so, we created 100 matrices where we simulated new straggler associations by matching each straggler with a new host from the adjacency matrix but different from the natural hosts of that mite species. Differences in the proportion of within-module stragglers between real and simulated matrices were evaluated using the function *prop.test* from the package *STATS* v3.4.3. Finally, the network was plotted using the plotweb function from *BIPARTITE v2.08* (Dorman et al., 2008).

Results

We collected a total of 3,477 bird's mite communities from which we successfully built 3,090 libraries, and we eventually obtained sequences from 1,130 bird's mite communities (25,540 individual mites), from 64 mite and 71 bird species, respectively. Notably, we found straggler mites in 84 bird's mite communities (1,228 individual mites), i.e., 8.1% of the communities and 4.8% of the individual mites studied. Straggling was not a restricted phenomenon, but involved 45.2% of bird and 53.9% of mite species, and 20.6% of the bird-straggler-mite associations were found more than once (Table 1). Also, in a 44.3% (N=35) of bird's mite communities where straggler mites were present and exoskeletons were preserved for morphological analyses (N=79), we found larvae or nymphal stages, and in a 45.6% (N=36) we found both males and females, supporting reproduction or potential for reproduction on that bird, respectively (Table S2, Supporting information).

Table 1. The number of bird's mite communities with straggler mites found in each bird species. Numbers between parenthesis indicate the total number of birds sampled for that bird species.

Bird species	Dolichodectes edwardsi	Dolichodectes hispanicus	Joubertophyllodes modularis	Monojoubertia microphylla	Proctophyllodes cetti	Proctophyllodes ciae	Proctophyllodes clavatus	Proctophyllodes cotyledon	Proctophyllodes doleophyes	Proctophyllodes lusciniae	Proctophyllodes mesocaulus	Proctophyllodes motacillae	Proctophyllodes musicus	Proctophyllodes pinnatus	Proctophyllodes rubeculinus	Proctophyllodes schwerinensis	Proctophyllodes stylifer	Proctophyllodes sylviae	Pteronyssoides parinus	Scutulanyssus obscuroides	Trouessartia bifurcata	Trouessartia inexpectata	Trouessartia minuscula	Trouessartia reguli	Trouessartia serrana	Trouessartia trouessarti
Acrocephalus arundinaceus (27)												1														
Acrocephalus melanopogon (16)				2								1														
Acrocephalus schoenobaenus (9)				2								1														
Acrocephalus scirpaceus (29)	2											1									1					
Carduelis carduelis (22)				2																						
Carduelis chloris (7)				2													1									
Carduelis citrinella (2)														1		1										
Cettia cetti (39)					1						1						5	1		1						
Emberiza cirlus (8)				6	2																					
Erithacus rubecula (95)							1														1					
Estrilda troglodytes (2)							1															1				
Ficedula hypoleuca (50)				2																						
Galerida cristata (3)		1																								
Hirundo daurica (1)				1																						
Hirundo rupestris (1)																				1						
Hirundo rustica (50)													1													
Lanius excubitor (2)													1		1											
Locustella luscinioides (12)				8																						
Luscinia megarhynchos (67)														1												1
Luscinia svecica (46)													1				1									
Muscicapa striata (22)				1																						
Oenanthe hispanica (2)									2																	
Oenanthe leucura (1)										1																
Oenanthe oenanthe (7)					1												1									
Phylloscopus bonelli (3)									1																	
Phylloscopus collybita (17)							1							1				1			3					
Phylloscopus trochilus (34)													1	1												
Regulus ignicapilla (2)																								1		
Saxicola torquatus (2)							1																			
Sylvia melanocephala (26)			1												4											
Turdus merula (19)																					1				2	

Excluding stragglers, most birds (94.6%) bore one (natural) mite species, 5.2% two, and only 0.2% had three mite species. In 69.1 % of the birds with stragglers, these were the only mite species. In the remaining 30.9%, stragglers shared the host with a non-straggler mite species, being stragglers coinhabiting a bird more frequent than expected by the proportion of bird's mite communities with two non-straggler mite species (i.e., 5.2 vs. 30.9%; $\chi^2 = 20.56$; df = 1; $P < 0.001$).

Overall, the average infrapopulation (i.e., all the mites of a particular mite species occurring in an individual host) size of stragglers was smaller than that of non-straggler species (Wilcoxon; $W = 43,042$, $P < 0.001$). However, in some samples, some straggler mites reached similar average intensities to non-straggler mites (Fig. 1). Among stragglers, bird's mite communities with reproductive stages (see above) showed higher intensity values (Wilcoxon; $W = 163$, $P < 0.001$).

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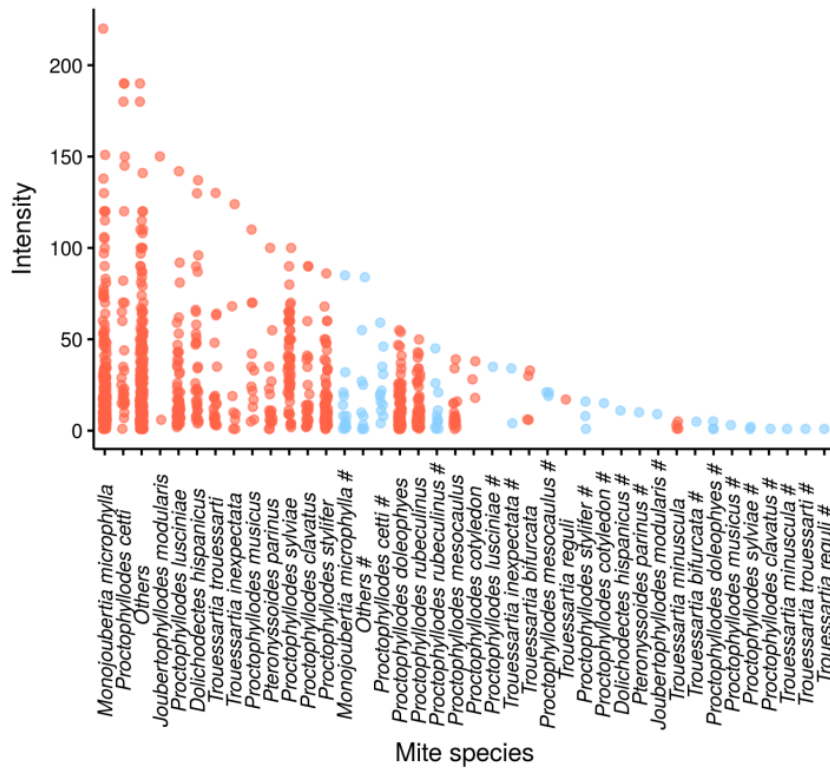
The minimum, mean and maximum genetic distances between sequences from straggler and non-straggler mite individuals showed different patterns among mite species. First, even maximum genetic distances between stragglers and non-stragglers mite individuals of the same species were lower than the mean smallest interspecific distances found for feather mites in Doña et al., (2015a) in all cases (Fig. 2). Second, in ten straggler species, we found that at least some straggler sequences were from a haplotype also found in the sequences of non-straggler individuals (i.e., min distance = 0). However, in 47.4 % of the mite species, we found differences in mean or maximum genetic distances above mean normal intraspecific distances when compared to non-straggler mites. Also, haplotype networks were overall more reticulated in these species than in mite species with lower genetic distances (Figs. 2; S1-S19, Supporting information).

FIGURE 2 Boxplots showing the genetic distances of straggler infrapopulations. Dashed gray lines show reference intra- and inter-specific thresholds for feather mites (Doña et al., 2015a). Haplotype networks belong to Proctophyllodes cetti (left) and Proctophyllodes rubeculinus (right).
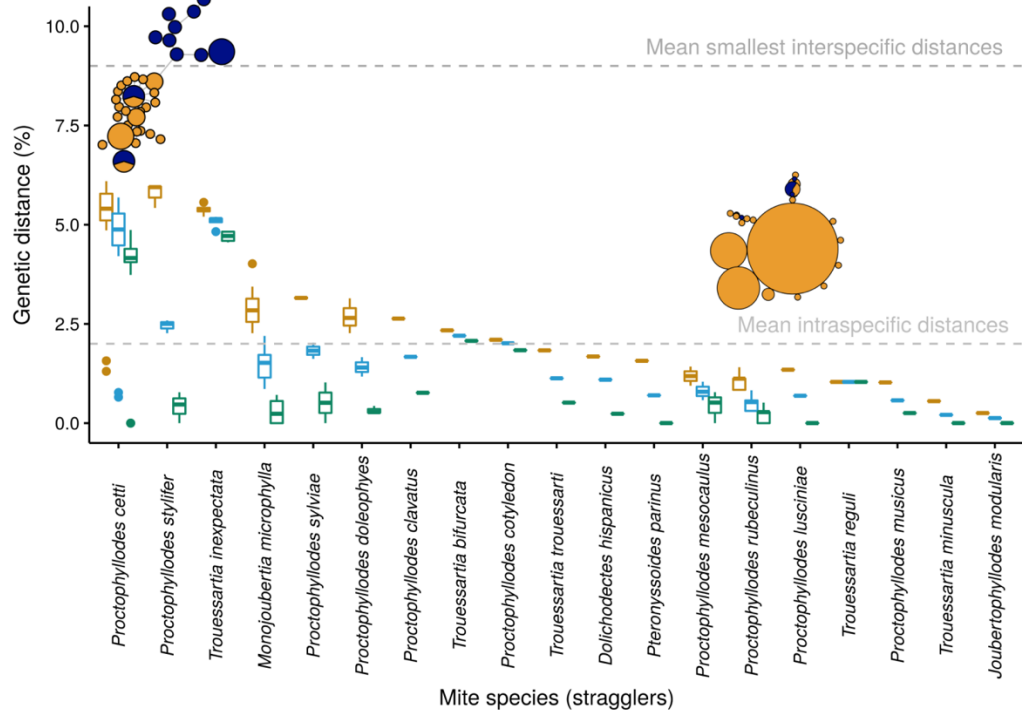
Straggler mites colonized hosts that were more distantly related than expected according to the relatedness of usual hosts of feather mite species in this study (Fig. 3 a, b; W= 476,650; P<0.001). The same result was found for the global database of bird-feather mite associations (Fig. 3 a, b; W= 101,250; P<0.001, Doña et al., 2016). However, there was no significant difference between the body mass of the usual and the unusual host of the same mite species (Fig. 4; Wilcoxon, W= 257,970, P=0.35).

Fig. 3. a) The probability that a pair of bird species sharing a feather mite species has a particular phylogenetic distance. Each line depicts probabilities of different mite subsets. Phylogenetic potential shows pairwise genetic distances between all hosts. Error bars represent confidence intervals ($\alpha=0.05$). b) Arcdiagram showing straggler mites and their association with usual hosts in a phylogenetic context.
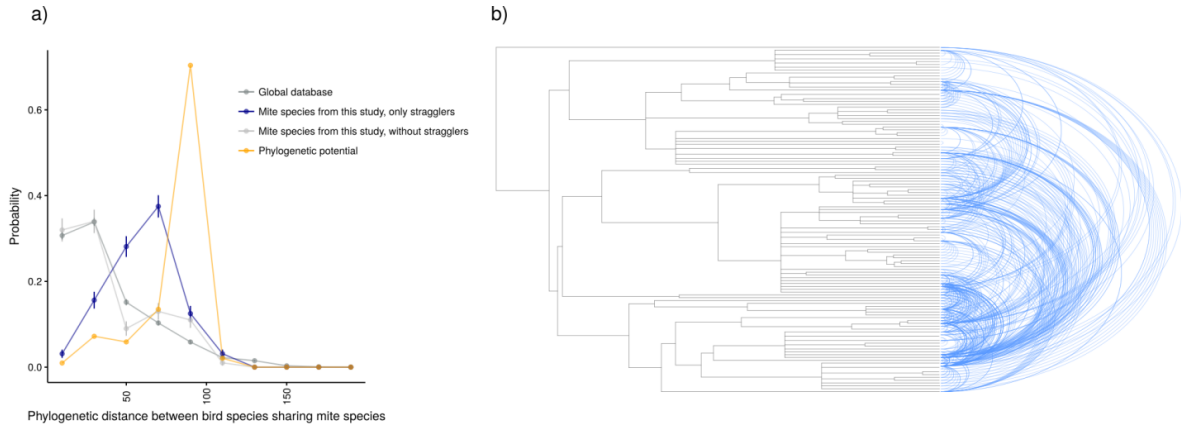
Fig. 4. Scatter plot showing the differences in body mass between hosts sharing a mite species, accounting for the phylogenetic relatedness. Black dots depict pairwise comparisons between usual hosts, and blue dots depict comparisons including unusual hosts. Points are horizontally jittered (2 points) to improve visibility.
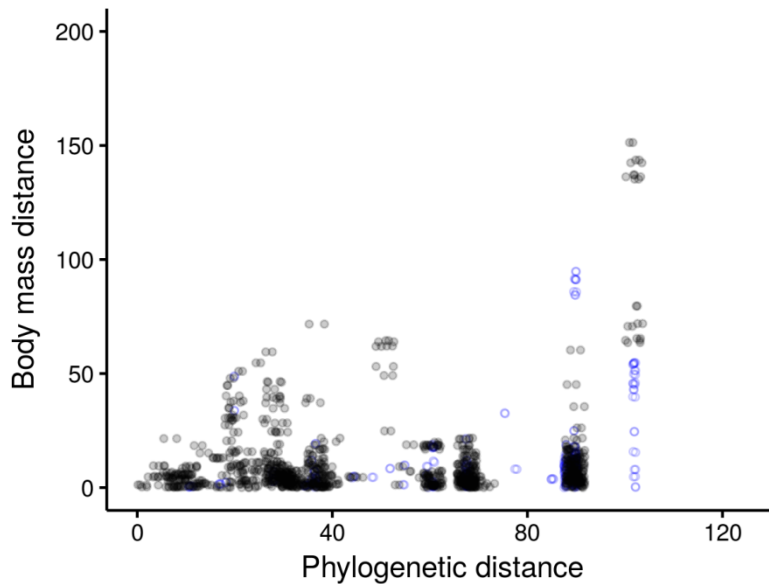
The bird-feather mite network was composed of 21 modules (Fig 5), with an average (min-max) of seven mite species (1-27) and nine (1-26) bird species per module. All modules were composed of hosts more closely related than expected by chance (mean (min, max) D = -

1.96 (-4.7, -0.17); and Pr (D = 1) = 0 in all cases; Table S3, Supporting information). Interestingly, we found that 26.5% of the straggler events were found within modules, a much higher frequency than the 5.9% expected by chance ($\chi 2$= 32.891; df = 1; P<0.001; Fig 5).

Fig. 5. Feather mites and birds ecological network. Color labels depict module composition (mites above, host birds below). Link colors represent feather mite module composition. Thicker dashed black lines represent stragglers found in the same module and thicker dashed gray lines represent stragglers found outside the module.
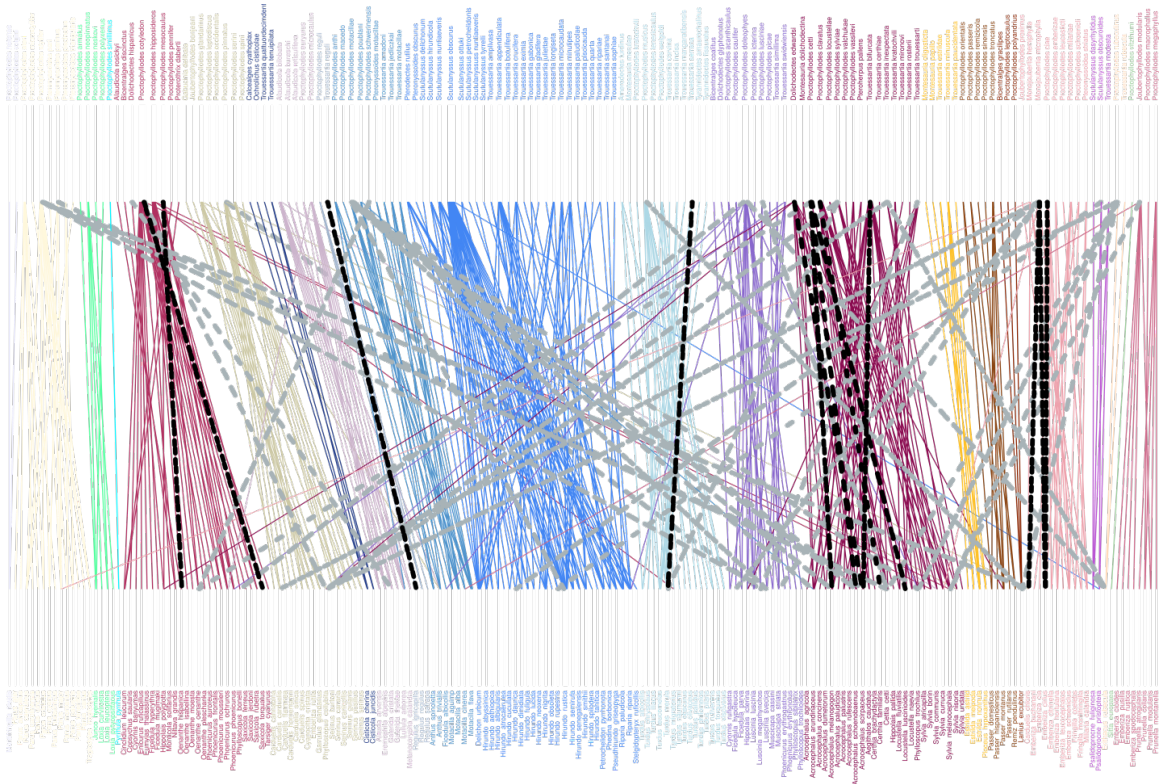
Discussion

Contrary to what was expected for these highly host-specific and specialized symbionts, we found a high prevalence of unexpected associations (i.e., stragglers; 8.1% of the infrapopulations and 4.8% of the individual mites), and even instances of ongoing host-switching. A rough calculation of straggler feather mites in European passerines shows the relevance of our result. A conservative estimation of population size for European passerine species is of ca. 10^9 bird individuals (BirdLife International, 2017). This, jointly with a conservative mean individual bird feather mite abundance of 10 mites per bird (Diaz-Real et

al., 2014) leads to 10^{10} feather mites living in European passerines. Therefore, the straggler prevalence reported here yields 10^8 individual birds with stragglers and 10^8 feather mite stragglers only for European passerines. Note, moreover, that this is a clear underestimation of the transmission of mites between bird species out of their usual host range. This is because (pure) stragglers, by definition, are sporadic, ephemeral, and mainly short-lived associations, and thus the occurrence of straggling in a given bird population is by definition underestimated with snapshot sampling of individual birds in a given moment in time. Moreover, we have found that unexpected associations were not random (as expected for pure stragglers) but constrained (see below) and that some even showed evidence of genetic differentiation. This strongly suggests that our straggling estimation, while being much higher than anticipated, is an underestimation, showing that feather mite transmission opportunity seems not to be a factor as limiting as previously thought, and alerting about the potential magnitude of straggling for other host-specific symbionts. In addition, our results encourage to focus on which are the mechanisms behind the high prevalence of straggling as feather mites do no transmit by phoresis neither are able to survive out of hosts.

Overall, these results at an ecological and microevolutionary scale help to explain the lack of bird-feather mites phylogenetic congruence at a macroevolutionary scale, and the power of major host-switches to trigger further diversification (Doña et al., 2017a; Doña et al., 2018a; Matthews et al., 2018). More importantly, our results go beyond explaining macroevolutionary patterns, given that the reported prevalence of straggling depict a highly dynamic scenario, where macroevolutionary patterns may be only the blurred echoes of ecological and microevolutionary processes, and thus demanding to focus on these low-scale dynamics.

Indeed, our results provide important hints about these processes. Interestingly, we found (Fig. 3) stragglers in hosts (i) that were more distantly related to the usual hosts than expected according to the phylogenetic host-specificity of usual bird-feather mite associations; (ii) partially overlapping with the longest phylogenetic distances reported for usual associations in Doña et al., (2018a), (iii) and much shorter than potential associations with other bird species found in the same localities (e.g., mite species coming from non-passerine birds of the study localities would have introduce hosts in the analysis which would have shown phylogenetic distances above 100 in Fig. 3). First, this shows that feather mites

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present a high phylogenetic host-specificity (Doña et al., 2018a) not because of a lack of transmission opportunities, but likely because of strong ecological filters. Also, this shows that while most stragglers would likely not persist much time in their new hosts, some may succeed (and in fact, we have found genetic evidence of ongoing host-switching). However, if they succeed, the comparison with usual associations strongly suggests that most of them would lead to host-shift speciation, thus reducing the host range of the (parent) mite species again.

Our results also advance in our understanding of the strong ecological filters encountered by stragglers. The most reasonable filters are the ones imposed by host morphology or other host traits with a strong phylogenetic signal that may explain the short phylogenetic distances between hosts occupied by feather mite species in their natural host range. Our results point to host morphological traits related to body-mass; given that we found that the body mass difference between straggler and natural hosts fell within the differences found between natural hosts (Fig. 3). Potential candidate traits that would merit further study are wing flight feather traits such as interbarb distance. Also, our results suggest that some of these filters may be not related to host morphology. This is because we found that stragglers coexisted with a non-straggler mite species in the same host more frequently than in usual bird's mite communities, thus suggesting that interspecific competition may preclude host range expansion (Johnson et al., 2009; Fernández-González et al., 2015; Doña et al., 2017b).

Overall, our results on stragglers, ongoing host-switching, and ecological filters depict a scenario highly compatible with a geographic mosaic of coevolution (Thompson, 1994, 2005; Poulin, 2010; Clayton et al., 2016; Ivens et al., 2016; Pinheiro et al., 2016), despite we did not investigate the selection mosaic per se. Also, in agreement with a geographic mosaic of coevolution, we found contrasting differences between mite species in the degree of genetic differentiation when comparing stragglers with non-stragglers mites (Fig. 2), which may reflect differences in the degree of gene flow and a continuum in the time of the separation from natural hosts (i.e., from pure stragglers to ongoing host-switches; Johnson et al., 2002; Nash et al., 2008). In this mosaic, each host inhabited by a mite species may impose different types of selection. In fact, we would expect differences in the type (and also in the intensity) of selection for mites according to the host inhabited. For instance, an

increase in mite body size favoring the match with the feather interbarb distance of one host would be maladaptive in other hosts (Dubinin, 1951; Gaud & Atyeo, 1996; Proctor & Owens, 2001). Studying mite morphology and fitness of same mite species in different host species would help in this direction.

The extent of straggling and host-switching reported here may seem low in comparison with dispersal potential of free-living organisms, compromising the feasibility of the proposed mosaic scenario. However, our previous studies on this system have shown that gene flow might be enough to maintain a low level of genetic differentiation between same mite species in different (usual) hosts, showing that gene flow may be enough for trait-remixing in a geographic mosaic scenario (Thompson, 1994, 2005; Nash et al., 2008, Doña et al., 2015a, Nash, 2008). Indeed, trait mismatching and local maladaptation are more likely when rates of gene flow are low (Nuismer et al., 2003). Thus, straggling and host-switching would allow the trait-remixing, infrequent/unstable associations may act as cold-spots, and frequent/stable associations as hot-spots of coevolution, as found in other less host-specific and specialized host-symbiont systems (Nuismer et al., 2003; Brockhurst et al., 2007; Nash et al., 2008). Future investigations on highly host-specific symbiont systems should integrate data on selection mosaics (i.e., experimental testing of the coevolutionary process, e.g., by performing experimental switches to hosts from the same and different network modules). These studies would allow ascertaining to which extent the functioning of the coevolutionary scenario of highly host-specific symbionts is analogous to that of a geographic mosaic of coevolution found in other systems in which populations are more connected.

Overall, our study depicts a more dynamic than foreseen scenario of host-symbiont associations in a highly host-specific symbiont system. This scenario is congruent with the recent results of the relevance and prevalence of host-shift speciation at a macroevolutionary scale (Doña et al., 2017a; Doña et al., 2018a; Matthews et al., 2018). Also, with recent evidence of host-switching in other host-symbiont systems (Ricklefs et al., 2004; Rivera-Parra et al., 2017; Bourguignon et al., 2018). More importantly, though, our results not only help to explain macroevolutionary patterns but have uncovered important pieces of a potential scenario of a geographic mosaic of coevolution (Thompson 1994, 2005; Nash, 2008; Clayton et al., 2016). This mosaic of coevolution may be played mainly within bird-

feather mite network modules, where host-symbiont, but also symbiont-symbiont interactions may be playing a central role.

The survey of straggling and host-switching dynamics at an ecological time is now more realizable than ever because of the advance on e-DNA methods, such as the DNA metabarcoding used in this study, which allows identifying and quantifying symbionts in a manner that up to now was inconceivable. Also, genomic studies now allow deepening in the population genomics of host-switches and would help to put host-switches in a historical context, helping to understand how they impact and have impacted speciation (Sweet et al., 2018b). Investigations in other host-symbiont systems with different properties combining these new technologies with traditional methods, such as controlled experimental host-switches in captivity, will not only improve our understanding of the eco-evolutionary scenario of host-shift speciation but will be decisive in the difficult task of integrating ecology and evolution to understand host-symbiont systems.

Acknowledgements

Appendix 1.

PCR cycles above routine numbers do not compromise high-throughput DNA barcoding results

Abstract

High-throughput DNA barcoding has become essential in ecology and evolution, but some technical questions still remain. Increasing the number of PCR cycles above the routine 20–30 cycles is a common practice when working with old-type specimens, which provide little amounts of DNA, or when facing annealing issues with the primers. However, increasing the number of cycles can raise the number of artificial mutations due to polymerase errors. In this work, we sequenced 20 COI libraries in the Illumina MiSeq platform. Libraries were prepared with 40, 45, 50, 55, and 60 PCR cycles from four individuals belonging to four species of four genera of cephalopods. We found no relationship between the number of PCR cycles and the number of mutations despite using a nonproofreading polymerase. Moreover, even when using a high number of PCR cycles, the resulting number of mutations was low enough not to be an issue in the context of high-throughput DNA barcoding (but may still remain an issue in DNA metabarcoding due to chimera formation). We conclude that the common practice of increasing the number of PCR cycles should not negatively impact the outcome of a high-throughput DNA barcoding study in terms of the occurrence of point mutations.

Introduction

High-throughput DNA barcoding (for single specimens; Shokralla et al. 2014, 2015; Toju 2015), as well as similar methods such as DNA metabarcoding (for mixed species samples; Taberlet et al. 2012) or amplicon metagenomics, combine DNA-based species identification using standardised markers (DNA barcoding, Hebert et al. 2003) with the power of high-throughput sequencing (HTS). These methods are powerful tools in life sciences research (Taberlet et al. 2012; Kress et al. 2015; Toju 2015), from studying century-old type specimens (Prosser et al. 2016), to assessing species composition of gut microbiota (Abdelrhman et al. 2016) from mixed samples.

Here, we focus on high-throughput DNA barcoding. This methodology overcomes some of the problems that currently limit DNA barcoding, such as the high DNA template concentration required for Sanger sequencing and the co-amplification of other DNA templates due to intrasample contamination, Wolbachia infection, gut contents, heteroplasmy, and pseudogenes. Moreover, high-throughput DNA barcoding reduces both per specimen costs and labour time by nearly 80%, thus allowing to be scaled up to deal with large-scale biodiversity monitoring projects (Shokralla et al. 2015; Cruaud et al. 2017).

However, even though high-throughput DNA barcoding is a promising method, some technical issues require further study. For example, some authors have explored the impact of the sequencing platform (Smith and Peay 2014), the polymerase used (Oliver et al. 2015; Brandariz-Fontes et al. 2015), the DNA barcode length (Hajibabaei et al. 2006; Doña et al. 2015), the library preparation method (Schirmer et al. 2015), the primers (Schirmer et al. 2015), the annealing temperature (Schmidt et al. 2013), or the phenomenon known as mistagging (Schnell et al. 2015; Esling et al. 2015) in DNA metabarcoding or amplicon sequencing. Recently, Geisen et al. (2015) and Díaz-Real et al. (2015) studied to what extent DNA metabarcoding produced quantitative (and not only qualitative) and reliable results in two groups of symbionts. Finally, several other papers have dealt with some of these issues

through bioinformatic analysis of the HTS reads (Caporaso et al. 2010; Coissac et al. 2012; Edgar 2013; Bokulich et al. 2013; Boyer et al. 2016).

Here, we focused on the number of PCR cycles used for library preparation. This is a technical issue that can potentially impact the biological conclusions of high-throughput DNA barcoding projects, but that has not yet been studied in detail. Increasing the number of PCR cycles above the normal 20–35 cycles (e.g., Shokralla et al. 2014, 2015; Carew et al. 2017) is a common practice: for example, when working with old-type specimens (Prosser et al. 2016), which provide small amounts of input DNA, or when the PCR is inefficient (e.g., Blaalid et al. 2013; Ellis et al. 2013; Carew et al. 2017). However, a large number of PCR cycles may entail the risk of increasing the number of artificial mutations on the output sequencing reads because of DNA polymerase errors and the amplification of these errors in subsequent PCR cycles (Cha and Thilly 1993; Hengen 1995; Casbon et al. 2011; Brandariz-Fontes et al. 2015). This is a potential major problem for high-throughput DNA barcoding because it can eventually distort, among others, genetic threshold-based species delimitation. Yet, to our knowledge, how these extra cycles affect DNA barcoding results has never been investigated.

To explore the consequences of the number of PCR cycles upon the number of artificial mutations, we extracted DNA from four different individuals belonging to four cephalopod species. From each of the four DNA samples, we prepared five high-throughput DNA barcoding libraries with different number of PCR cycles: from 40, i.e., roughly 20 cycles higher than regular numbers, to 60, as done commonly when dealing with problematic samples. After sequencing the 20 libraries using the Illumina MiSeq platform, we studied the relationship between the number of PCR cycles and the number of mutations present in the MiSeq reads. Our results show that, for a number of cycles between 40 and 60, there is no relationship between the number of PCR cycles and the number of mutations, with the number of reads with mutations being very low. Therefore, we conclude that a number of PCR cycles as high as 60 does not compromise the success of a high-throughput DNA barcoding project in terms of the occurrence of point mutations.

Materials and methods

Four ethanol-preserved tissues obtained from different cephalopod species belonging to the orders Octopoda, Oegopsida, and Sepiida were analysed (see sample IDs and cephalopod species in Table 1). Species were identified according to morphology and DNA bar coding (Fernando Fernández-Álvarez, personal communication). The genetic p-distances between the selected individuals were between 80.1 and 85.7 for the cytochrome c oxidase subunit I gene (COI) used in this study.

Total DNA was extracted from each individual using the NZY- Tissue gDNA Isolation Kit (NZYTech). DNAs were quantified with the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) and used as input for the preparation of the libraries.

We followed a standard Illumina library preparation protocol. In brief, we amplified the COI region (i.e., the standard animal barcode, Hebert et al. 2003) and included the Illumina specific adapters and indices by following a two-step PCR approach, slightly modified from Lange et al. (2014). For the sake of clarity, we refer to these PCRs as PCR1 and PCR2.

PCR1 primers were LCO1490 and HCO2198 (Folmer et al. 1994), which proved successful in a previous study in which the same specimens were DNA barcoded (Fernando Fernández-Álvarez et al., personal communication). Oligonucleotide tails bearing the Illumina sequencing primers were attached to the 5′ ends of primers LCO1490 and HCO2198. PCR2 was carried out with tailed primers that bear the indices and adapters and anneal to the Illumina sequencing primers (see Fig. 1 for a schematic representation of the binding process).

FIGURE 1 Schematic representation of the primers used for PCR1 and PCR2 (see main text). The positions of the Illumina adapters, indices, and sequencing primers are also shown. Note that primers are not drawn to scale.
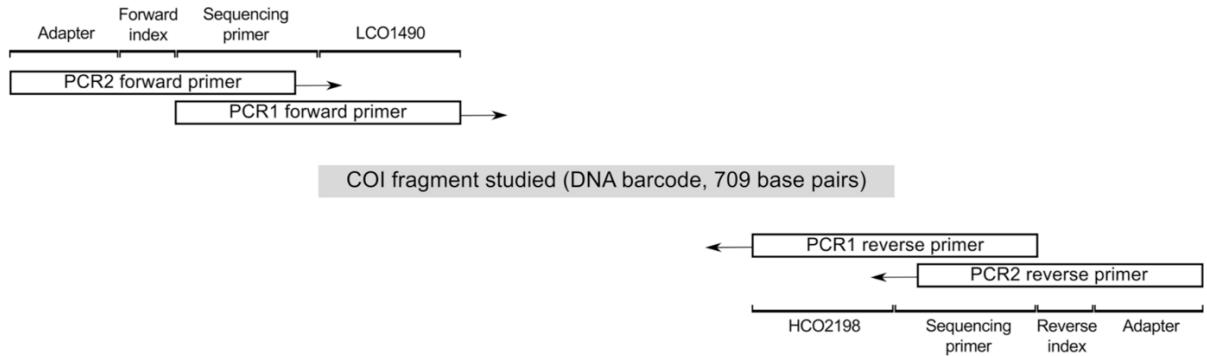
PCR1 was carried out using 25 ng of total DNA in a final volume of 25 μL containing 6.50 μL of Supreme NZYTaq Green PCR Master Mix (NZYTech) (nonproofreading polymerase; error rate of 1×10^{-5} according to the manufacturer), 0.5 μM of each primer, and PCR-grade water up to 25 μL. The thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 35, 40, 45, 50, or 55 cycles (see Fig. 2) of denaturation at 95 °C for 30 s; annealing at 53 °C for 30 s; extension at 72 °C for 45 s; and a final extension step at 72 °C for 10 min. The products of PCR1 were purified using the SPRI method (DeAngelis et al. 1995), with Mag- Bind RXNPure Plus magnetic beads (Omega Biotek). The purified products were loaded in a 1% agarose gel stained with GreenSafe (NZYTech) and visualised under UV light.

PCR2 was carried out using 2.5 μL of the purified PCR1 products, and the same conditions as for PCR1 except for the number of cycles, which was set to five (Fig. 2) and the annealing temperature (60 °C). The products obtained were purified following the SPRI method as indicated above. Then, the purified products were loaded in a 1% agarose gel stained with GreenSafe (NZYTech) and visualised under UV light. All samples yielded libraries of the expected size.

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FIGURE 2 From each cephalopod sample, five different high-throughput DNA barcoding libraries were constructed and sequenced in the Illumina MiSeq platform. In each of these five libraries, the number of PCR cycles during PCR1 was different (35, 40, 45, 50, and 55 cycles).
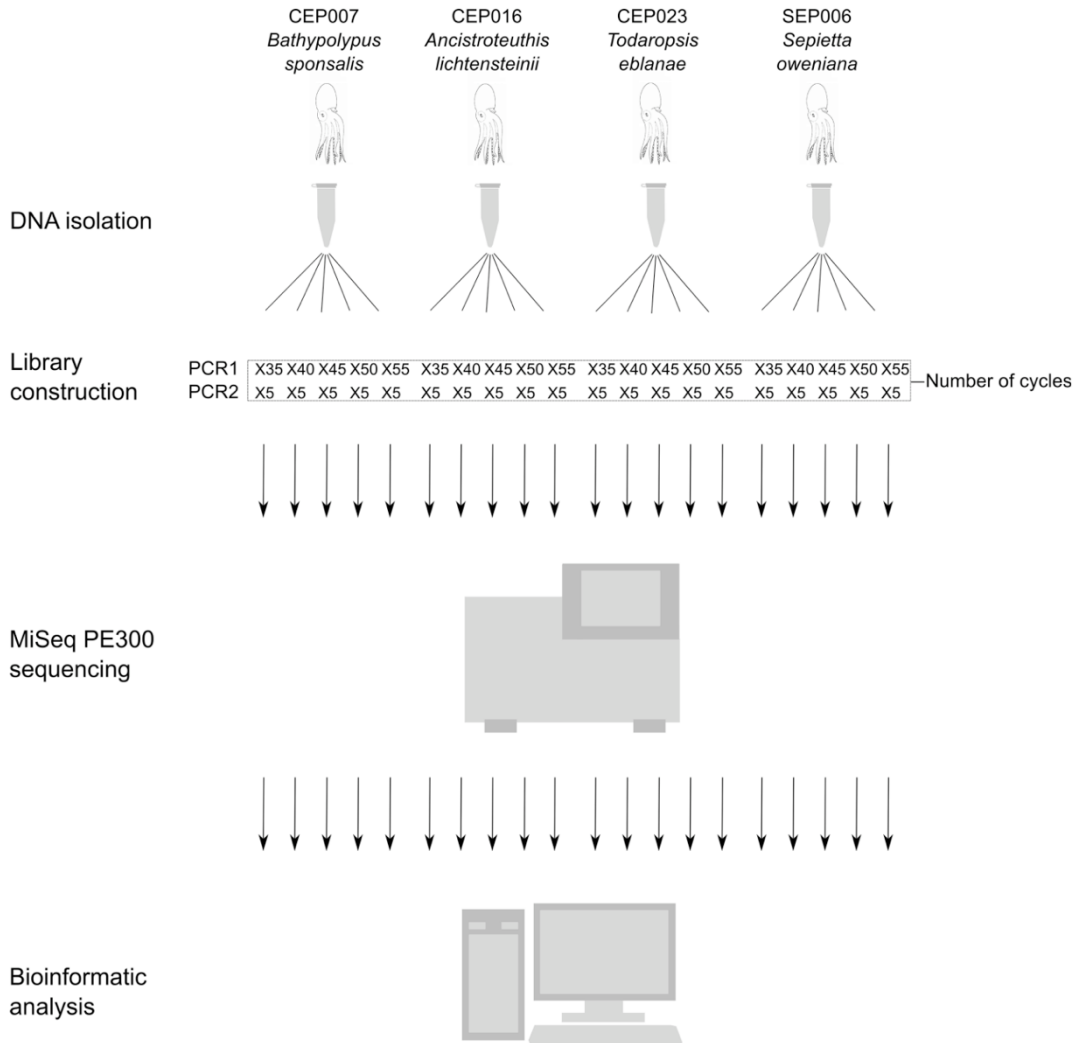
Libraries were quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) and pooled in equimolar amounts. The pool was sequenced in a fraction of a 600-cycle run (MiSeq Reagent Kit v3; PE300) of an Illumina MiSeq sequencer along with a PhiX library used to increase sequence diversity of the overall library, in Macrogen (Seoul, Korea).

FASTQ files were demultiplexed using *RTA 1.18.54* (Illumina) and checked with *FastQC 0.11.3* (http://www.bioinformatics.babraham. ac.uk/projects/fastqc/). Then, they were quality-trimmed using very conservative parameters in *Trimmomatic 0.36* (Bolger et al. 2014) with the option SLIDINGWINDOW:1:30. SLIDINGWINDOW starts scanning at the 5´ end and clips the read once the average quality within the window falls below a threshold (Trimmomatic Manual 0.32). We set the size of the window to 1 and the quality threshold to 30 (Phred Quality Score). Therefore, when the quality of a single nucleotide fell below a Phred Quality Score of 30, the read was clipped from this position to the 3´ end. We used these very conservative parameters to make sure that the mutations observed in the sequencing results were due to PCR errors and not to sequencing errors. The quality of the resulting files was checked again with FastQC.

Quality-trimmed FASTQ files were imported into *Geneious 8.1.6* (http://www.geneious.com, Kearse et al. 2012). Each pair of R1 and R2 files were set as paired reads to improve the mapping. A map- to-reference analysis was carried out with the *Geneious* mapper using relaxed parameters (maximum number of mismatches per read, 25%; minimum overlap identity, 80%) to allow potentially mutated reads to map. The DNA barcode sequences from the four cephalopod specimens were set as references (DDBJ/EMBL/ GenBank accession numbers KX078469–KX078472). The results of the map-to-reference analysis were inspected manually to verify that the reads of each library mapped to the correct reference sequence. We obtained 20 assembly files corresponding to the four species by the five PCR treatments.

Regions including the first 50 nucleotides of the mapped R1 and R2 reads (starting immediately after the primer annealing region) were aligned in each assembly with *Muscle* (Edgar 2004) as implemented in *Geneious 8.1.6.* We selected these two 50-nucleotide

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regions because such read length accumulated the maximum number of reads after passing the quality threshold (see above); using larger regions would have reduced the sample size and, therefore, the statistical power of the analysis. Reads were trimmed to the same length to simplify later bioinformatic analyses.

For each alignment file, we calculated the number of mutations per read by comparing every read against the consensus sequence. The consensus sequences obtained from the FASTA files of the same species were identical between them (regardless of the number of PCR cycles) and they were also identical to the corresponding COI sequences available in DDBJ/EMBL/GenBank. For this, we used a custom developed *R* function (R Core Team 2016) to calculate the number of mutations by multiplying the pairwise genetic p-distance by the total length of our reads. The function treated insertions and deletions (indels) as single mutational steps and the genetic p-distance was calculated with the *dist.dna* function (raw model) from the *ape 3.4 R* package (Paradis et al. 2004). Then, we ran a Poisson generalised linear mixed model (GLMM) on the entire resulting data set (*glmer* function from package *lme4 1.1-12*; Bates et al. 2015). We considered the number of mutations as the response variable, the number of cycles as the predictor variable, and the species as a random factor. We confirmed assumptions underlying GLMMs by exploring regression residuals for normality against a Q-Q plot.

Finally, to make sure that the PCR1 reaction was still functioning after 55 cycles (i.e., that the emergence of new artificial mutations was still possible), qPCRs were performed in all four samples with the same parameters as in PCR1, but with 60 cycles to cover the whole range of our experiment. The resulting fluorescence versus number of cycles plots were visually analysed, confirming that the reaction was still taking place after 55 cycles.

Results

Due to the stringent quality-filtering, only 2.26% of the raw reads were used for the statistical analyses (see supplementary material, Table S1). The average quality of both the raw and

quality-trimmed reads, as measured with FastQC, is available in the supplementary material, Fig. S1.

We detected mutations in 4176 out of the 69 792 reads analysed (i.e., 5.98%), which passed the quality-filtering step, mapped to the correct reference sequence, and were located within the 50-nucleotide stretches after the primer annealing regions.

The number of mutations was consistent across species and the maximum number of mutations per read was three along different treatments (Fig. 3; Table 1). Accordingly, we found no effect of the number of cycles on the number of mutations (Fig. 3; slope ± SE = 0.0002 ± 0.0024, Z = 0.096, P = 0.923).

Table 1. Percentage of reads with 0, 1, 2, or 3 mutations relative to the reference sequence.

Library ID	0	1	2	3	Number of reads
CEP007 (*Bathypolypus sponsalis*)	94,055	5,772	0,167	0,004	22.105
CEP016 (*Ancistroteuthis lichtensteini*)	94,169	5,607	0,222	0	14.408
CEP023 (*Todaropsis eblanae*)	93,409	6,37	0,198	0,022	22.637
SEP006 (*Sepietta oweniana*)	95,019	4,839	0,14	0	10.642

FIGURE 3 Number of mutations relative to the reference sequence observed in each PCR treatment. (a) Bathypolypus sponsalis. (b) Ancistroteuthis lichtensteini. (c) Todaropsis eblanae. (d) Sepietta oweniana.
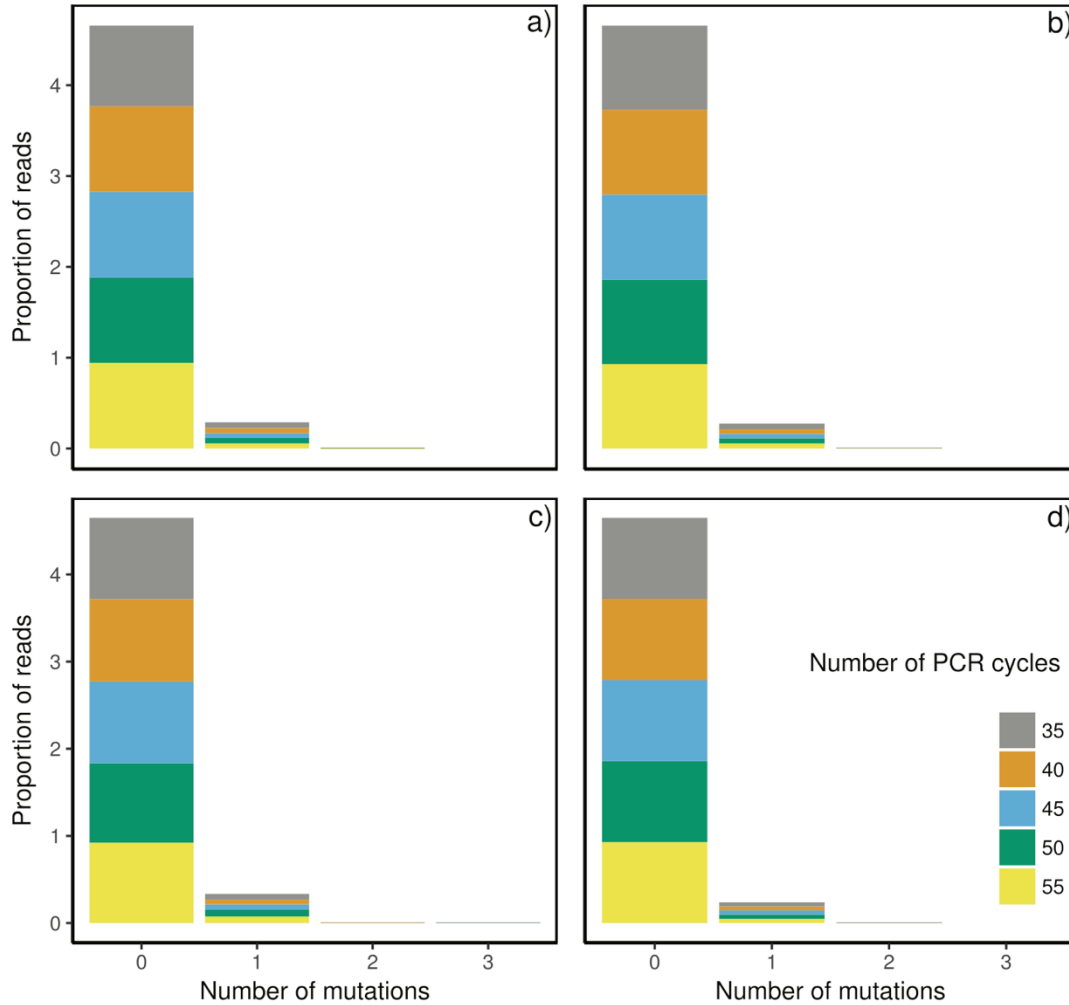
Discussion

In this work, we investigated whether increasing the number of PCR cycles during library preparation produces a higher number of mutations that could eventually impact the outcome of a high-throughput DNA barcoding study. We demonstrated that even for a high number of cycles (60, i.e., up to 55 cycles for PCR1 and five additional cycles for PCR2) the

number of reads with mutations remained very low despite using a non-proofreading enzyme and despite the potential occurrence of heteroplasmy (which would increase the number of mutated positions when compared to the reference sequence). However, we only analysed two regions of 50 nucleotides each from the COI animal DNA barcode, whereas different genomic regions may impose different error rates to DNA polymerase (e.g., Arezi et al. 2003). Nevertheless, the lack of effect we found in these regions with high sequence quality by experimentally increasing the number of PCR cycles indicates that PCR cycles might have negligible impacts on point mutations and subsequent taxonomic assignment.

Some DNA metabarcoding-specific technical issues can arise by an increase in the number of PCR cycles, and thus require further study. For instance, chimeras are hybrid amplicons that can be formed during a PCR when an aborted extension product from an earlier cycle functions as a primer in a subsequent PCR cycle (Haas et al. 2011). Chimeras inflate diversity in an artificial manner and should be carefully taken into account. In this work, chimeras were not an issue because we prepared our libraries using DNA from individual specimens (i.e., high-throughput DNA barcoding libraries). However, the formation of chimeras has been found to be correlated with the number of PCR cycles and to the consumption of the primers (Wang and Wang 1996; Qiu et al. 2001; Thompson et al. 2002). Fortunately, several bioinformatic tools have been developed to deal with chimeras and thus their impact can be greatly reduced (Edgar et al. 2011, Haas et al. 2011, Coissac et al. 2012, Boyer et al. 2016). Thus, even though our results hold for DNA metabarcoding studies in terms of point mutations, the formation of chimeras at high PCR cycles is a separated problem that should be considered in DNA metabarcoding studies.

Overall, our results show that increasing the number of PCR cycles above routine levels during library preparation is not risky for high-throughput DNA barcoding studies, in terms of the amount of point mutations produced by polymerase errors even when a non-proofreading enzyme is used. Therefore, this strategy can be safely followed with little amounts of input DNA or when there are mismatches in the primer annealing regions that make the PCRs inefficient.

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Acknowledgements

Appendix 2.

A new feather mite of the genus *Dolichodectes* (Astigmata: Proctophyllodidae) from *Hippolais polyglotta* (Passeriformes: Acrocephalidae) in Spain

Abstract

A new feather mite species, *Dolichodectes hispanicus* sp. n. (Astigmata: Proctophyllodidae), is described from the Melodious Warbler *Hippolais polyglotta* (Vieillot) (Passeriformes: Acrocephalidae) in Spain. The new species is closest to the type species of the genus, *D. edwardsi* (Trouessart, 1885) from the Grear Reed-Warbler Acrocephalus arundinaceus (Linnaeus) (Acrocephalidae). Adults of *D. hispanicus* differ from those of *D. edwardsi* by dimensional characteristics, in particular, by having shorter aedeagus that does not extend to the anal suckers in males and shorter hysteronotal shield in females. Tritonymphs of *D. hispanicus* are much more distinctive and differ from those of *D. edwardsi* by having the prodorsal shield covering all the prodorsum, the hysteronotal shield occupying about three quarters of the hysterosoma, and idiosomal setae h3 being filiform. The morphological description of the new species is augmented by sequence data from the mitochondrial cytochrome c oxidase subunit I gene fragment (COI).

Introduction

The feather mite genus *Dolichodectes* Park et Atyeo, 1971 (Astigmata: Proctophyllodidae) was established in a generic revision of the subfamily Pterodectinae (Park and Atyeo 1971), and to date it has included eight species (Mironov and Fain 2003, Mironov et al. 2010, 2012). In the subfamily Pterodectinae, this genus and four more genera, *Alaudicola* Mironov, 1996, *Anisodiscus* Gaud et Mouchet, 1957, *Montesauria* Oudemans, 1905 and *Pedanodectes* Park et Atyeo, 1971, constitute the *Montesauria* generic complex, which can be considered derived pterodectines of the Old World (Mironov 2009). Within this complex, the genus *Dolichodectes* is clearly characterised by having a greatly elongated body in both sexes, and strongly elongated opisthosomal lobes, setae ps3 situated posterior to the anal suckers and bases of setae g and ps3 arranged in a long rectangle in males (Park and Atyeo 1971, Mironov 2009). As for most pterodectine genera, excluding some specialised genera associated with hummingbirds, representatives of this genus inhabit vanes of the primary and secondary feathers of the wings and tail, where they are located in corridors on the ventral surface.

Mites of the genus *Dolichodectes* have been recorded from representatives of seven passerine families distributed in the Old World: Acrocephalidae, Phylloscopidae, Platysteiridae, Muscicapidae, Turdidae, Ploceidae (Passeroidea) and Monarchidae (Corvoidea). Of eight species previously included in this genus, its type species, *Dolichodectes edwardsi* (Trouessart, 1885) associated with warblers of the genera *Acrocephalus* Naumann et Naumann (Acrocephalidae) and *Phylloscopus* Boie (Phylloscopidae), is widely distributed in the Old World. *Dolichodectes bifurcatus* Mironov, Literák, Nguen et Čapek, 2012 is known from *Copsychus malabaricus* (Scopoli) (Muscicapidae) in southeastern Asia, and the six remaining species are recorded only from passerines in Africa (Trouessart 1885, Gaud and Mouchet 1957, Gaud and Till 1961, Mironov 1996, Mironov et al. 2010, 2012). Additionally, Atyeo (1973) reported one undescribed *Dolichodectes* species from *Hippolai calligata* (Lichtenstein) (Acrocephalidae) in southeastern Asia. Hernandes & Valim (2006) con- structed a key to species of *Dolichodectes* and described a new species, *D. neotropicus* Hernandes

& Valim, 2006, from a cotinga host (Passeriformes: Cotingidae) in Brazil. Subsequently, these authors (Valim and Hernandes 2009) found out that this species actually belonged to the generic complex of *Pterodectes* Robin, 1877 and it was removed to a separate genus *Berladectes* Valim et Hernandes, 2009.

In the present paper, we describe a new *Dolichodectes* species from the melodious warbler *Hippolais polyglotta* (Vieillot) (Acrocephalidae) in Spain. The morphological description of a new species is complemented by the sequence data on the mitochondrial cytochrome c oxidase subunit I gene fragment (COI) (Doña et al. 2015).

Materials and methods

Mite specimens used for description were collected from live birds in Spain for our general project 'Quantitative barcoding of birds' feather mites: taxonomy meets ecology' (started in 2012), one of the main goals being to create a barcoding library of feather mites from European passerines (Doña et al. 2015). Mites were manually collected from the feathers using a flattened preparation needle or a cotton swab impregnated with ethanol and preserved at -20 °C in tubes with 96% ethanol. Then mites were mounted on slides for identification in Hoyer's medium according to standard techniques for small mites (Krantz and Walter 2009).

Five mite specimens from different bird individuals were subjected to DNA extraction. After extraction, the exoskeletons were also mounted on slides. DNA extraction, PCR amplification and sequencing were conducted using the protocol described in another paper (Doña et al. 2015). Pairwise genetic distances were calculated in *MEGA 6* (Tamura et al. 2013) using the K2P model (Kimura 1980). In addition, we used the Refined Single Linkage (RESL) algorithm to calculate the Barcode Index Number (BIN) (Ratnasingham and Hebert 2013). The mite samples from *Hippolais polyglotta* were mostly represented by tritonymphs and just a few adults were found; this could be probably explained by the fact that collections from this host were made during migration seasons.

Description of a new species is given according to the modern schemes for pterodectine mites (Hernandes and Valim 2006, Mironov et al. 2008, 2010, 2012, Valim and Hernandes 2010). General morphological terms and leg chaetotaxy follow Gaud and Atyeo (1996); the idiosomal chaetotaxy also follows these authors with corrections to coxal setation added by Norton (1998). All measurements are in micrometres (μm). Measuring techniques used for particular structures were recently described in Mironov et al. (2008, 2012) and Mironov and González-Acuña (2011). Specimens of *Dolichodectes edwardsi*, used for comparison in the differential diagnosis, were collected from its type host, the great reed-warbler *Acrocephalus arundinaceus* (Linnaeus) (Acrocephalidae), in the same locality where the type material of the new species was collected (Doñana National Park, Huelva, Spain).

The taxonomic system and scientific names of birds follows Clements et al. (2014). Specimen deposition is indicated by the following abbreviations: EBD – Estacion Biológica de Doñana (Seville, Spain), ZISP – Zoological Institute of the Russian Academy of Sciences (Saint Petersburg, Russia).

Results

Family **Proctophyllodidae** Trouessart et Mégnin, 1884

Subfamily **Pterodectinae** Park et Atyeo, 1971

Genus *Dolichodectes* Park et Atyeo, 1971

Dolichodectes hispanicus sp. n. Figs. 1–3, 4A, B, D

ZooBank number for species: urn:lsid:zoobank.org:act:CF347684-71FA-430A-9A55-30E1D553CF2B

Male (Figs. 1, 3A–D) (holotype, measurement for 1 paratype in parentheses). Length of idiosoma 515 (520), width 165 (170), length of hysterosoma 355 (360). Prodorsal shield: anterior margin with triangular rostral process, anterolateral extensions connected to bases of epimerites Ia, lateral margins without incisions around scapular setae, posterior margins slightly convex, posterior part with transverse striae near posterior margin, length along mid-

line excluding rostrum 130 (125), width at posterior margin 110 (115) (Fig. 1A). Setae *ve* represented by alveoli. Bases of scapular setae *se* separated by 70 (68). Humeral shields absent. Setae *cp* and *c2* situated on soft tegument. Setae *c3* lanceolate, 22 (21) × 7 (8). Hysteronotal shield: greatest length from anterior margin to lobar apices 360 (365), width at anterior margin 135 (140), anterior margin shallowly concave, anterior angles rounded, anterior half of this shield with transverse striae, area from level of trochanters IV to bases of opisthosomal lobes with small longitudinal lacunae. Opisthosomal lobes nearly 3 times longer than wide at base, lateral margins at level of setae *h2* noticeably convex, posterior end of each lobe with small acute extension. Terminal cleft a narrow almost parallelsided slit, lateral margins touching, length 78 (84), greatest width in anterior half 5–8. Supranal concavity short, with heavily sclerotised margins. Setae *f2* and *ps2* situated at same transverse level. Setae *h1* situated at level of supranal concavity, approximately equidistant from levels of setae *f2* and setae *e2*. Setae *h3* lanceolate with acute tips, situated approximately equidistant from lobar apices and bases of setae *h2*, length 57 (55), greatest width 12 (13); setae *ps2* 60 (65) long, slightly extending beyong to lobar apices; setae *ps1* filiform, minute, about 10 long. Distance between bases of dorsal setae: *c2:d2* 130 (125), *d2:e2* 93 (90), *e2:h2* 73 (66), *h2:h3* 27 (31), *d1:d2* 45 (55), *e1:e2* 22 (24), *h1:h2* 44 (45), *ps1:h3* 11 (12), *h2:h2* 55 (62), *h3:h3* 27 (25), *ps2:ps2* 62 (66).

Epimerites I fused into a Y, sternum about 1/2 of total length of epimerites, posterior end of sternum with transverse extensions connected to medial part of epimerites II (Fig. 1B). Coxal fields I, II without large sclerotised areas. Coxal fields I–IV closed. Rudimentary sclerites rEpIIa absent. Coxal fields IV with large sclerotised areas at bases of trochanters IV. Genital arch of moderate size, 22 (23) long, 30 (28) wide; basal sclerite of genital apparatus shaped as inverted trapezium. Aedeagus 82 (85) long, extending to midlevel of anal suckers. Genital papillae poorly distinct, situated at midlevel of genital arch. Paragenital apodemes fused to each other by their medial parts into a long median sclerite, anterior branches of these apodemes fused with inner margins of epimerites IIIa and their posterior branches fused with epimerites IV. Genital shields, epimerites IVa, posterior branches of paragenital apodemes

and shield–like areas of coxal fields IV fused altogether to form almost complete sclerotised oval surrounding genital apparatus. Genital shields short and not fused to each other at midline of body. Setae *4b* on anterior branches of paragenital apodemes, setae *4a* on posterior branches of paragenital apodemes, setae *g* on genital shields. Opisthoventral shields wide, fused together by wide transverse bridge immediately posterior to anal opening; anal field flanked posteriorly and laterally by opisthoventral shields and transverse bridge. Anal suckers 15 (13–15) in diameter, corolla without indentations. Setae *ps3* situated on anterior margin of transverse band connecting opisthoventral shields. Setae *4b* situated slightly posterior to level of setae 3a. Distance between ventral setae: *4b:4a* 70 (68), *4a:g* 38 (30), *g:ps3* 53 (52), *ps3:ps3* 40 (42), *ps3:h3* 88 (86).

FIGURE 1 Dolichodectes hispanicus sp. n., from Hippolais polyglotta (Vieillot), male. **A** – dorsal view; **B** – ventral view. Remark: setae sR of trochanters III are deliberately represented as both present and absent
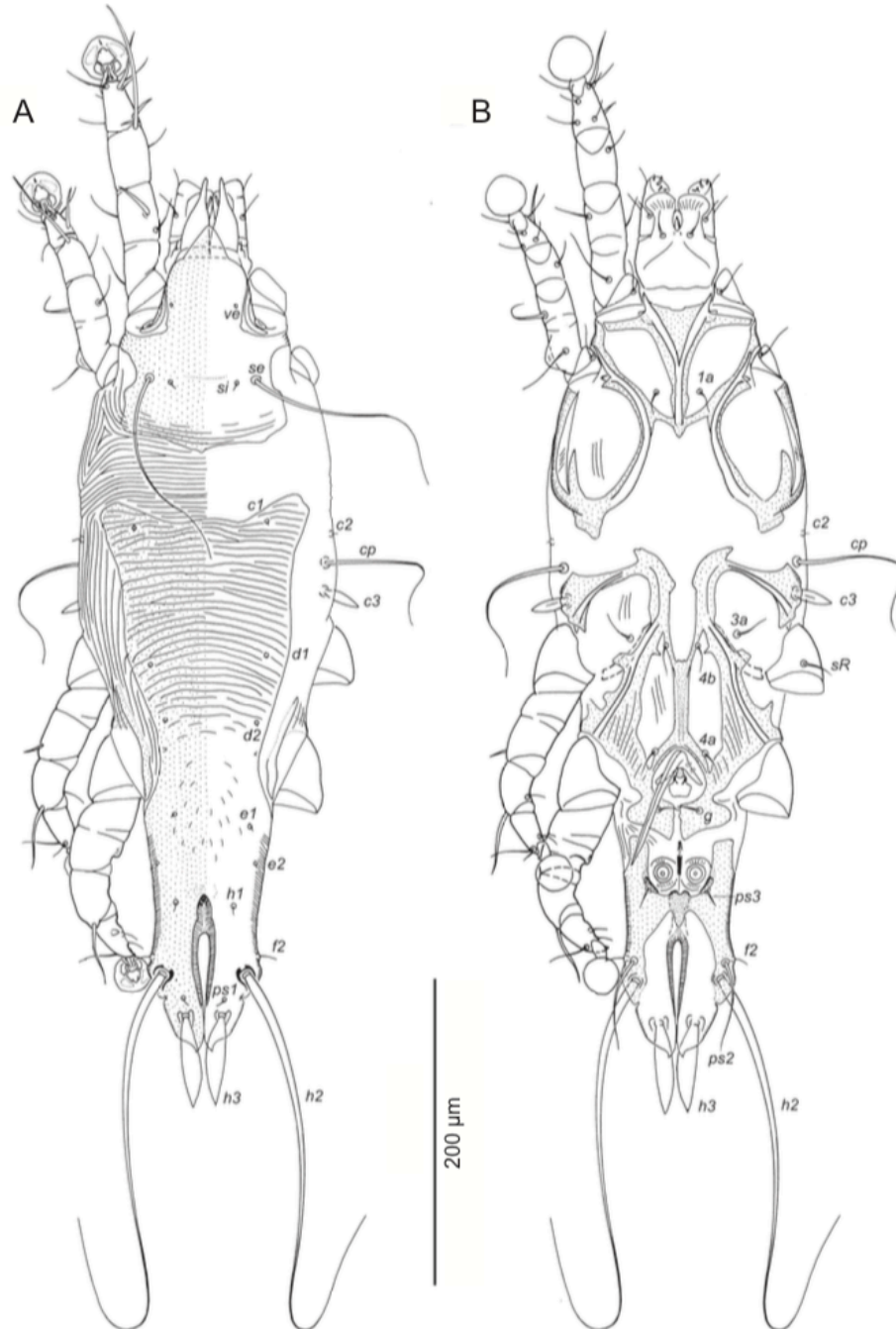
Legs I longer and thicker than legs II, femora II with narrow ventral crests, other segments of these legs without processes (Fig. 3A, B). Solenidion σ1 of genu I 24 (22) long, situated in proximal part of segment; genual setae *cGI*, II, *mG* I, II filiform. Genu IV with narrow, heavily sclerotised ventral crest (Fig. 3D). Setae *sR* of trochanters III absent (in holotype) or present (in paratype). Solenidion ω1 of tarsus II elongate, extending to midlevel of ambulacral disc; seta d of tarsus II half as long as corresponding seta f. Seta d of tarsus III much shorter than corresponding setae *f*. Tarsus IV 30 (31) long, with small apical claw-like process; seta d hemispherical, with thick walls, situated in proximal part of this segment; seta e indistinct. Solenidion ϕ of tibia IV extending to midlevel of ambulacral disc. Length of solenidia: ω1I 14 (13), ω1II 23 (26), ϕI 88 (80), ϕII 53 (48), ϕIII 38 (40), ϕIV 42 (37).

   Female (Figs. 2, 3E–G) (range for 4 paratypes). Length of idiosoma 520–550, width 200–210, length of hysterosoma 355–370. Prodorsal shield: anterolateral extensions narrow and free from epimerites Ia, lateral margins with deep incision at level of scapular setae, posterior margin slightly convex, length along midline 122–130, width at posterior margin 120–125, posterior part with transverse striae (Fig. 2A). Setae ve represented by alveoli. Bases of setae se separated by 77–80. Humeral shields absent. Setae *cp* and *c2* situated on soft tegument. Setae *c3* lanceolate, 22–24 × 7–8. Anterior and lobar parts of hysteronotal shields completely separated dorsally from each other by narrow transverse band of soft tegument, but connected ventrolaterally. Anterior hysteronotal shield noticeably enlarged in anterior part, anterior margin convex, posterior margin shaped as recurved bow, length 265–280, width at anterior margin 150–155; anterior two thirds of this shield with dash-like transverse striae. Length of lobar region 85–90, width 90–93, anterior margin medially convex. Terminal cleft narrow, with lateral margins slightly divergent, 58–62 long, about 10 wide at level of lobar apicest. Supranal concavity absent. Setae *f2* present. Setae *h1* situated on anterior margin of lobar shield. Setae *h2* spindle-like, 52–55 long, 7–8 wide. Setae *ps1* approximately equidistant from inner and outer margins of opisthosomal lobes. Setae *h3* filiform, 12–15 long, about 1/6th length of terminal ap- pendages. Distance between dorsal

setae: *c2:d2* 125–133, *d2:e2* 115–120, *e2:h2* 45–50, *h2:h3* 43–50, *d1:d2* 50–57, *e1:e2* 38–42, *h1:h2* 15–18, *h2:ps1* 20–22, *h1:h1* 33–35, *h2:h2* 65–68.

FIGURE 2. *Dolichodectes hispanicus* sp. n., from *Hippolais polyglotta* (Vieillot), female. **A** – dorsal view; **B** – ventral view. Remark: setae *sR* of trochanters III are deliberately represented as both present and absent.
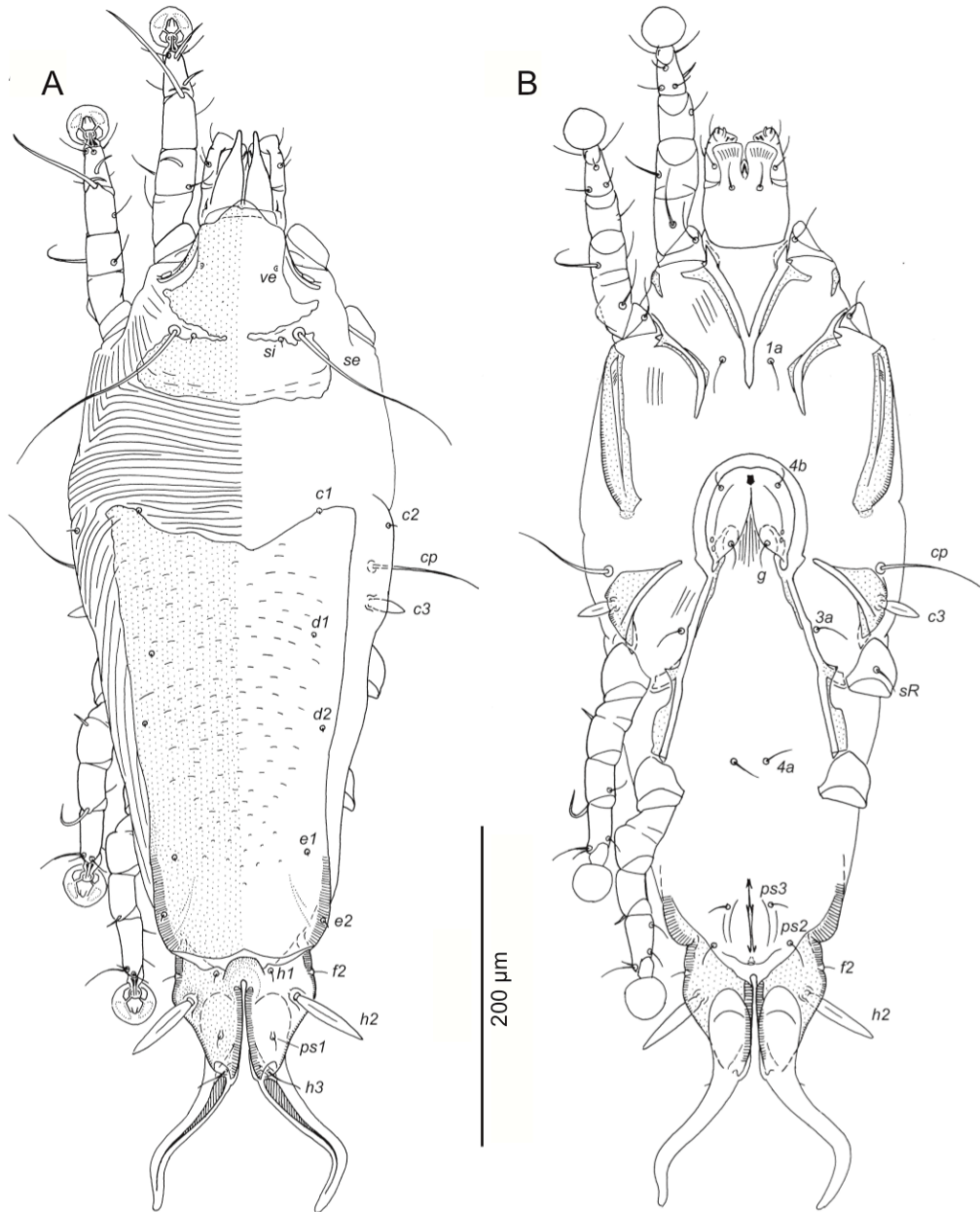
Epimerites I fused into a Y, sternum about 1/3 of total length of epimerites (Fig. 2B). Lateral parts of coxal fields I, II without wide sclerotised areas. Epimerites IVa absent. Translobar apodemes of opisthosomal lobes wide, not fused to each other anterior to terminal cleft. Copulatory opening situated immediately posterior to anal opening. Primary spermaduct with punctuated enlargement in most proximal part, secondary spermaducts 30–33 long (Fig. 3G). Distance between pseudanal setae: *ps2:ps2* 45–50, *ps3:ps3* 22–25, *ps2:ps3* 20–23.

Legs I, II subequal, femur II with narrow ventral crest, other segments of these legs without processes. Solenidion σ1 of genu I 15–17 long, situated closer to anterior margin of segment. Genual setae *cG*I, II, *mG*I, II filiform. Genu IV with small dorsal inflation. Setae *sR* of trochanters III absent (in 3 paratypes) or present (in 1 paratype). Setae d of tarsi II–IV much shorter than corresponding setae f. Solenidion ϕIV about 1/4 of corresponding tarsus (Figs. 3F). Length of solenidia: $\omega 1$I 15–16, $\omega 1$II 16–18, ϕI 68–73, ϕII 52–57, ϕIII 24–26, ϕIV 9–11.

FIGURE 3 Dolichodectes hispanicus sp. n., from Hippolais polyglotta (Vieillot), details. **A–D** – legs I–IV of male, respectively; E, F – legs III and IV of female, respectively; G – spermatheca and spermaducts. Abbreviations for Fig. 3G: co – copulatory opening, hs – head of spermatheca, pd – primary spermaduct, sd – secondary spermaduct.
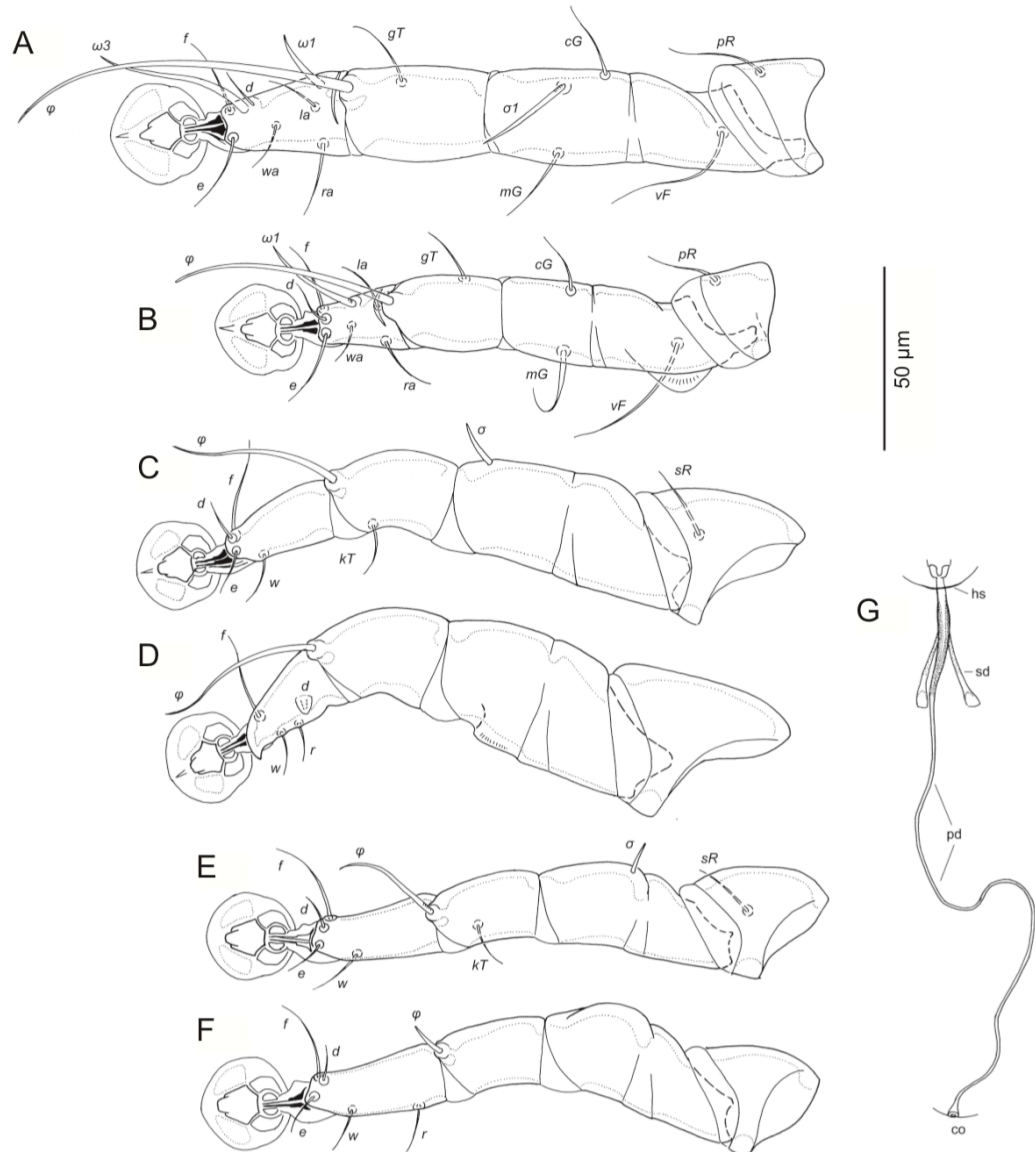
Tritonymph (Fig. 4A, B, D) (range for 13 paratypes). Length of idiosoma 480–510, width 160–175, length of hysterosoma 295–310. Prodorsal shield occupying almost all prodorsum and split by T-shaped furrow into three parts, one anterior and two posterior (Fig. 4A), surface without ornamentation, total length of this shield 150–160, width at level of posterior parts 135–150. Setae *ve* represented by alveoli. Bases of setae *se* separated by 78–86. Humeral shields absent. Setae *cp* and *c2* situated on soft tegument. Setae *c3* lanceolate, 16–18 × 7–8.

Prodorsal and hysteronotal shields separated from each other by sparsely striated area of soft tegument 60–80 long. Hysteronotal shield with widely rounded anterior margin, with anterior end almost extending to level of setae *c3*, length from anterior margin to lobar apices 245–260, width at level of setae *e2* 100–110, surface without ornamentation, supranal concavity present. Opisthosomal lobes long; terminal cleft between them narrow, with margins almost touching, 60–68 long. Hysteronotal setae *c1*, *c2*, *d1*, *d2* situated on soft striated tegument, remaining hysteronotal setae on hysteronotal shield. Setae *d2* situated at level of trochanters III. Setae *h2* spindle-like, 30–35 × 5–6. Setae *h3* situated near lobar apices, filiform, 70–80 long. Distance between dorsal setae: *c2:d2* 78–85, *d2:e2* 90–105, *e2:h2* 50–55, *h2:h3* 53–60, *d1:d2* 40–50, *e1:e2* 40–48, *h1:h2* 22–26, *h2:ps1* 35–40, *h1:h1* 38–42, *h2:h2* 65–73.

Epimerites I fused into a Y, sternum about 1/3 of total length of epimerites (Fig. 4B). Epimerites IIa present, long, each split into 3–4 longitudinal sclerites. Inner ends of epimerites IIIa close to each other, with longitudinal sclerotised bands of irregular form; length and contour of these bands variable, sometimes but not always extending to bases of setae 4b (Fig. 4B,D) . Epimerites IVa present, poorly sclerotised.

FIGURE 4 Tritonymphs of Dolichodectes species. *A, B, D* – *Dolichodectes hispanicus* sp. n., from *Hippolais polyglotta* (Vieillot), *C, E* – *Dolichodectes edwardsi* (Trouessart, 1885); *A, C* – dorsal view; *B* – ventral view; *D, E* – coxal fields III and IV. Remark: setae sR of trochanters III in *D.* hispanicus are deliberately represented as both present and absent.
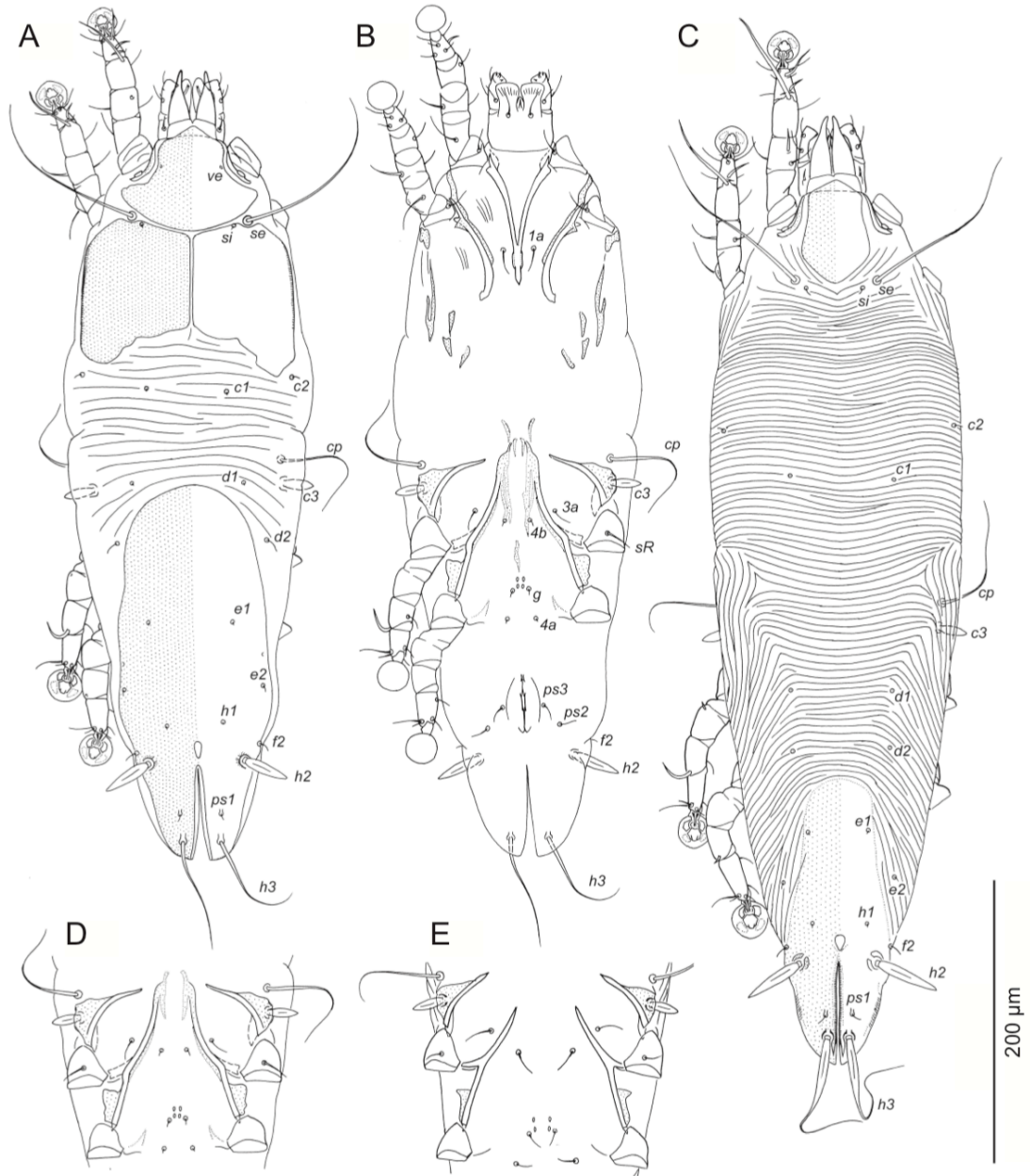
Form and setation of legs I, II as in female, except solenidion σ 1 of genu I reduced to a button-like structure. Solenidion σ of genu III absent. Setae *sR* of trochanters III absent (in 7 paratypes) or present (in 6 paratypes). Setae *d* of tarsi II–IV much shorter than corresponding setae *f*. Solenidion ϕ of tibia IV absent. Length of tibial solenidia: ϕ I 33–36, ϕ II 30–33, ϕ III 11–13.

T y p e   h o s t : Melodious warbler *Hippolais polyglotta* (Vieillot) (Passeriformes: Acrocephalidae)

T y p e   l o c a l i t y : Spain, Huelva, El Rocío, Doñana National Park, Manecorro, 37°07'21''N; 6°29'27''W.

D a t e   o f   c o l l e c t i o n : 15 October 2011,collected by R. Jovani.

T y p e   m a t e r l a l : Male holotype; 1 male, 4 female and 13 tritonymphal paratypes.

D e p o s l t l o n   o f   t y p e   m a t e r l a l : Male holotype (ZISP 20908), 1 male paratype (ZISP 20909), 4 female paratypes (ZISP 20904–20907), 13 tritonymphal paratypes (ZISP 20901–20903, 209010–20919).

A d d l t l o n a l   m a t e r l a l : one tritonymph (EBD1284ART) from *H. polyglotta*, Spain, Huelva, El Rocío, Doñana National Park, Manecorro, 37°07'21''N; 6°29'27''W, 5 May 2011, collected by R. Jovani; one tritonymph (EBD1286ART) from same host and location, 11 May 2011, collected by R. Jovani; one tritonymph (EBD1287ART) from same host and location, 12 May 2011, collected by R. Jovani; one tritonymph (EBD1289ART) from same host, Spain, Valencia, Tabarca, 38°09'21''N; 0°28'25.4''W, 4 May 2011, collected by A. Álvarez, one tritonymph (EBD1290ART), from same host and location, Spain, Valencia, Tabarca, 4 May 2011, collected by A. Álvarez.

R e p r e s e n t a t l v e   s e q u e n c e s : 602 bp fragment of the COI gene; GenBank accession Nos KP193461 (specimen EBD1284ART), KP193462 (EBD1286ART), KP193463 (EBD1287ART), KP193460 (EBD1289ART), KP193459 (EBD1290ART).

E t y m o l o g y : The specific epithet refers to the country of finding.

Differential diagnosis. The new species *Dolichodectes hispanicus* sp. n. is most similar to *D. edwardsi* described from the great reed-warbler *Acrocephalus arundinaceus* (Acrocephalidae) in having the following set of features (Trouessart 1885). In both sexes, the distance between the prodorsal and hysteronotal shields is relatively long, at least half as long as the length of the prodorsal shield; in males, coxal fields II are closed, opisthosomal lobes are acute apically; in females, the anterior margin of the hysteronotal shield is concave, setae h3 are short and do not exceed 1/4 the length of the terminal appendages.

Dolichodectes hispanicus differs from *D. edwardsi* by the following features: in males, the aedeagus is 78–84 μm long and extends to the midlevel of anal suckers (Fig. 1B), length of the terminal cleft is 82–85 μm. In females, the anterior hysteronotal shield is shorter and relatively wid- er (the greatest length 265–280 μm, ratio of the length to greatest width 1.7–1.8), length of idiosoma is 520–550 μm.

In males of *D. edwardsi* (n = 10), the aedeagus is 95–100 μm long and slightly extends beyond the posterior margin of anal suckers, the terminal cleft is 100–110 μm long. In females (n = 10), the anterior hysteronotal shield is longer and narrower (the greatest length 285–315 μm, ratio of length to greatest width is 2.0–2.2), length of idiosoma is 560–610 μm.

Whilst adults of *D. hispanicus* and *D. edwardsi* are very similar in their general appearance and differ from each other by mensurative characters, the morphological difference between tritonymphs of these species is much more conspicuous. In tritonymphs of *D. hispanicus*, the prodorsal shield covers almost the entire prodorsum and is split by T-shaped furrow into 3 pieces (one anterior and a pair of posterior plates), the hysteronotal shield extends to the level of subhumeral setae *c3*, transverse striae on the area between the prodorsal and hysteronotal shields are sparse, setae *h3* are filiform, solenidion σ1 of genu I is reduced to a small button, epimerites IIIa extend to the level of tips of epimerites III and bear longitudinal sclerotised bands, and epimerites IIa are present and split into several longitudinal sclerites (Fig. 4A,B,D).

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In tritonymphs of *D. edwardsi*, the prodorsal shield is represented by an entire plate covering only the median part of the prodorsum anterior to scapular setae, the hysteronotal shield extends to the level of trochanters IV, striation between dorsal shields is as dense as in adults, setae *h3* are spindle-shaped basally, solenidion $\sigma 1$ of genu I is normal setiform, the tips of epimerites IIIa are simple and do not extend to the level of epimerites III, and epimerites IIa are strongly reduced (Fig. 4C,E).

References DNA sequences. The average genetic distance among all specimens, in which sequences were examined, was 0.6% (SE 0.2). All nucleotide substitutions were synonymous. All sequences were identified as belonging to the same Barcode Index Number (BIN; ACR0931).

Remark. It is interesting to note an unstable state of the trochanteral seta *sRIII* in *Dolichodectes hispanicus* of adults and in tritonymph. In pterodectines of the *Pterodectes* generic group, the presence or absence of this seta is stable in species, and a state of this character is usually a good diagnostic feature for a genus. This character varies among species only in the genus *Montesauria* Oudemans, 1905 (Mironov et al. 2008, 2012, Mironov and Tolstenkov 2013).

Appendix 3.

The complete mitochondrial genome of the feather mite *Trouessartia rubecula*

Jablonska, 1968 (Astigmata: Analgoidea: Trouessartiidae)

Abstract

We assembled and annotated the complete mitochondrial genome of *Trouessartia rubecula*, the first feather mite complete mitochondrial genome from the largest feather mite superfamily Analgoidea (ca. 1150 spp). The mitogenome was composed of 13 protein, 17 tRNA, and 2 rRNA-coding genes and was 14,125 bp in length.

Feather mites (Acariformes: Astigmata: Analgoidea and Pterolichoidea) are the most common, abundant, and diverse ectosymbionts of birds (Doña et al. 2016). *Trouessartia rubecula* is a feather mite species which inhabits the flight feathers of European robins *Erithacus rubecula* (Doña et al. 2016). In this study, we present the complete mitochondrial genome of *T. rubecula*, which is the first feather mite complete mitochondrial genome from the superfamily Analgoidea.

Total genomic DNA was extracted using the MicroSpin kit (Real) from 30 *T. rubecula* individuals sampled from a single individual of *E. rubecula* at Corterrangel (Huelva, Spain) (37° 56' 14.1'' N, 6° 36' 00.2'' W). The DNA sample was submitted to the Novogene Bioinformatics Institute (Beijing, China) for library preparation and sequencing in a lane of an Illumina HiSeq 4000 PE150.

After performing a quality filtering step with *Trimmomatic 0.33* (Bolger et al. 2014), the reads were de-novo assembled using *ABySS 2.0.2* (Simpson et al. 2009). A 14.38kb contig which showed 77% of nucleotide identity to the mitochondrial genome of *Dermatophagoides pteronyssinus* (GenBank accession number: EU884425.1) was found.

The *MITObim software 1.9* (Hahn et al. 2013) was used to verify the reconstructed sequence using 2500bp of the *ABySS* contig as seed. A contig of 14.59 kb was obtained and circularized using the script circules (https://github.com/ chrishah/MITObim). Finally, the COI gene was placed at position 0 using *Geneious 10.2.2* (Kearse et al. 2012).

The *MITObim* contig was kept for downstream analyses (GenBank accession number: MH208456). The final length of the mitochondrial genome was 14.13 kb.

MITOS 2 (Bernt et al. 2013) was used to annotate protein, tRNA, and rRNA-coding genes. The protein-coding regions were manually validated using the *ORFfinder* tool (https://www.ncbi.nlm.nih.gov/orffinder/).

The mitogenome was composed of 13 protein, 17 tRNA, and 2 rRNA-coding genes. The 12S rRNA and 16S rRNA genes were 623 and 680 bp, respectively. The base composition was 28.53% A, 44.74% T, 10.24% C, and 16.49% G. The protein-coding sequence length was 10,797bp, encoding 3599 amino acids.

Most of the tRNA coding genes showed TV-replacement loops and their lengths varied from 53bp to 61bp (Klimov and OConnor 2009). tRNA-Ala, tRNA-Glu, tRNA-Ile, tRNA-Tyr, and tRNA-Val genes could not be predicted. Although the lack of certain tRNA coding genes has been previously observed in the Acaridae family (Yang and Li 2015), further research will be needed for a better reconstruction of the tRNAs of *T. rubecula*.

A maximum-likelihood phylogeny was inferred (Figure 1). In brief, we downloaded all available whole mitochondrial genomes of astigmatan mite species (plus an outgroup from Mixonomata) from the NCBI GenBank database (accession date: 2 April 2018). Mitochondrial genomes were aligned using *MAFFT v7.222* (Katoh et al. 2002), and the alignment was trimmed using *Trimal v1.4* (Capella-Gutiérrez et al. 2009). We inferred the tree using *IQ-TREE* (Nguyen et al. 2015) and *ModelFinder* (Kalyaanamoorthy et al. 2017) was used to find the optimal evolution model. Overall, the phylogenetic relationships found in this study were congruent with previous studies on the phylogeny of these mites (Klimov and OConnor 2013).

Figure 1. Phylogram based on the mitogenome sequences of Trouessartia rubecula (MH208456; this study) and eight other Astigmata mites (plus an outgroup from Mixonomata). The following mitochondrial genomes were used (accession numbers are in parentheses): Tyrophagus longior (NC_028725), Tyrophagus putrescentiae (NC_026079), Ardeacarus ardeae (KY352304), Dermatophagoides pteronyssinus (EU884425), Psoroptes cuniculi (NC_024675), Caloglyphus berlesei (NC_024637), Aleuroglyphus ovatus (KJ571488), Histiostoma blomquisti (NC_031377), and Steganacarus magnus (NC_011574), which was used as outgroup (Dabert et al. 2010). The phylogenetic tree was estimated from 500 bootstrap (BS) replicates in IQ tree. BS support values are indicated at each node and the scale bar indicates nucleotide substitutions per site.

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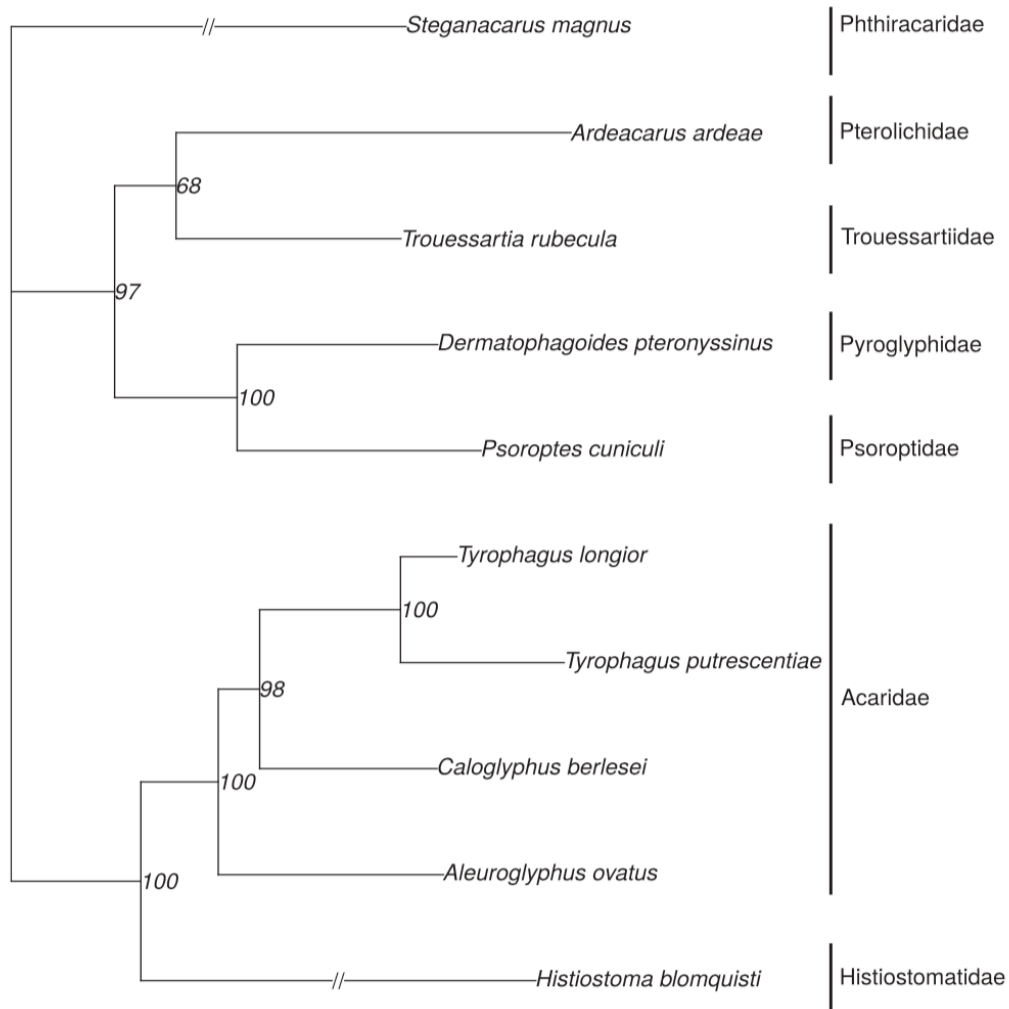
Appendix 4.

Opening the Doors of Parasitology Journals to Other Symbionts

Intimate symbiotic relationships between species (e.g., between a larger 'host' and a smaller 'symbiont') span the range from mutualism to parasitism. The nature of a symbiotic relationship is not an intrinsic trait of the species involved, but rather the outcome of their interaction. Many symbiont species move along the mutualism–parasitism continuum depending on the environmental conditions (Bronstein, 1994). For instance, defensive symbionts act as mutualists when they clean hosts of parasites, but they may harm their hosts when there are no parasites to clean (Hopkins et al. 2017). Similarly, parasites can become mutualists under some conditions, as exemplified by the Drosophila symbiont, Wolbachia (Weeks et al. 2007). Also, lineages often move along this continuum in evolutionary time (Weinstein & Kuris, 2016). However, boundaries between scientific disciplines are not so permeable.

Parasitology is the leading specialized discipline in symbiosis research, and the recent review by Hopkins et al. (2017) in *Trends in Parasitology* on defensive symbionts is a good example of the benefits parasitology can reap from integrating knowledge from other symbionts (Vannier-Santos & Lenzi, 20101; Jovani, 2003). Opening the doors of parasitology journals to other symbionts would be a decisive first step for parasitologists to fully embrace the study of other symbionts.

There is a paradox in the study of parasitology. For many species of symbionts it is difficult to tell whether they are harmful to their host (i.e., parasitic) until detailed research is done. Therefore, parasitologists studying an apparently parasitic species (e.g., feather mites living on birds) typically are not able to publish their work in a parasitology journal if the research concludes that they are mutualists. In studies of more complex contexts, such as the gut microenvironment, it makes no sense to study only parasitic species because all components of the gut flora, and the interactions between them, should be taken into account. This blurs the limits of parasitology and illustrates our point that parasitologists should embrace other symbionts.

Integrating knowledge, concepts, and experimental and statistical tools among researchers studying symbionts should also be encouraged. For instance, studies on host–

symbiont systems alert us to the potential risks of using defensive symbionts as biocontrol agents (as claimed in Hopkins et al. 2017). This is because current knowledge shows that even highly host-specific symbionts are able to switch among hosts (Clayton et al. 2016; Hoberg & Brooks, 2015), and host-switching dynamics are related to factors other than the nature of the host–symbiont relationship (Clayton et al. 2016). Thus, symbionts used for biological control of one species may shift to nontarget hosts. This is potentially risky if we consider the possibility of a change in the mutualistic–parasitic interaction continuum under the new ecological scenario after a host-switch, either benefiting or harming unwanted (nontarget) hosts.

Given the biological reasons for, and the strategic benefits of, merging symbiont research outlined above, we advocate opening parasitology journals to studies on other symbionts – and not only when they directly help us to understand (currently) 'true' parasites. Then the question that arises is how to optimize the specialization–general relevance trade-off needed in any scientific discipline. Perhaps we could learn from the Royal Entomological Society that "exists to promote the dissemination of knowledge in all fields of insect science", but its Ecological Entomology journal has the policy to publish "top-quality original research on the ecology of insects and related invertebrate taxa", thus embracing knowledge from associated groups. In our opinion, similarly, opening the doors of parasitology journals to other symbionts would be fruitful for parasitology itself, and the study of symbionts as a whole.

Discussion

Feather mites have remained intractable for large-scale eco-evolutionary studies because of the lack of proper methodologies. In this thesis, resources and molecular tools for the study of feather mites have been developed. Then, using these tools, essential aspects of the evolutionary ecology of feather mites which are relevant to understand their diversification have been investigated. Lastly, using this knowledge and tools, the diversification of feather mites at a macro- and microevolutionary scale has been investigated.

Resources and molecular tools for the study of feather mites:
Chapter 1 reports a comprehensive and global catalog of bird-feather mite associations previously scattered in articles, checklists, zoological surveys and books. Also, the dataset was taxonomically curated, becoming the first and most updated global taxonomic reference for this group of symbionts. Also, it will be periodically updated to include new data appearing in the literature.

This database represents an extremely useful resource not only for the advance of feather mite knowledge but also for the study of symbionts as a whole. Similar data in other groups of symbionts (e.g., fleas, feather lice; Medvedev, 1997a, b; Price et al. 2003) have been extensively used for studying a wide array of topics, such as the factors influencing the diversity of symbionts (Krasnov et al. 2004), testing the Szidat's rule (Krasnov et al. 2016), or the role of competition in the evolution of host generalism (Johnson et al. 2009). First examples of the use of this database can be found in chapters 3-8. Also, it has been used in a study where it was studied the response of symbiont species to climate change (Carlson et al. 2017).

Chapter 2 reveals DNA barcoding as a powerful tool for feather mite studies. In this chapter, the accuracy of DNA barcoding and minibarcoding for feather mites' studies was

tested. In addition, the first DNA barcode library of feather mites was compiled. Also, a procedure to improve the robustness of genetic thresholds was developed and used to establish a fine-tuned genetic threshold for species delimitation within this group (Doña et al. 2015a).

By using DNA barcoding under an integrative taxonomic approach (Schlick-Steiner et al. 2010), three putatively cryptic species were found (Doña et al. 2015a). Also, this study led to the description of a new feather mite species, Dolichodectes hispanicus (Appendix 2; Mironov et al. 2012), and it has facilitated further molecular studies (e.g., Doña et al. 2015b; Doña et al. 2017b; Doña et al. 2018a). DNA barcoding methodologies are advancing fast, and now the field is moving towards the extended barcode concept (i.e., the use of whole genomes instead of concrete molecular markers; Coissac et al. 2016). In this thesis, a complete mitochondrial genome of a feather mite species has been assembled and annotated (Appendix 3), and mitochondrial PCGs were used in chapter 7 (Doña et al. 2017b). In addition, a nuclear genome has been assembled (but not included in this thesis) and is currently being improved with long-read sequencing data.

Overall, these tools overcome one of the major problems when studying feather mites, the difficulty (sometimes impossibility) in species-level identification even for highly specialized taxonomists. In addition, it significantly improves feather mite's taxonomy as this methodology allows to robustly implement molecular data in species descriptions, and thus to follow an integrative taxonomic approach (Schlick-Steiner et al. 2010). Lastly, similarly to other global initiatives in different groups (e.g., birds, Kerr et al. 2007; Fishes, Ward et al. 2009; Plants, Zúñiga et al. 2017), the transversal utility of this DNA barcoding approach reassures extending the current DNA barcode library from the European to a global scale.

Lastly, chapter 3 presents a quantitative Illumina DNA metabarcoding pipeline . The pipeline is based on the utility of DNA barcoding (chapter 2) and enables for the first time large-scale inventories of feather mites, while it is extendable to other similar symbionts. Also, it allows retrieving intensity data for all the feather mite species present in a host simultaneously. For instance, this pipeline was used to investigate the eco-evolutionary

scenario of host-shift speciation in chapter 8 (Doña et al. in prep). In addition, it also allowed to delineate nine putative new feather mite species and to obtain useful genetic data of 31,566 individual mites. These next-generation inventorying tools will contribute significantly to the future of ecological and evolutionary studies (Taberlet et al. 2012a, b). However, despite its current utility, like other DNA metabarcoding pipelines, the evaluation of this pipeline will need of constant fixation and improvements (Elbrecht et al. 2017). For instance, Appendix 1 shows that the high number of PCR cycles used in this (and similar) pipelines when working with such small symbionts does not compromise the results. However, the DNA extraction strategy, as well as the primers used, may need further improvement in the future. Current DNA isolation strategy used is based on the HotSHOT method (Truett et al. 2000). It preserves exoskeletons, and thus it is extremely useful in circumstances where further taxonomic work may be needed. However, it yields a little amount of DNA which in many cases is below the minimum required for high-throughput sequencing. Accordingly, dividing the mite samples to use more aggressive DNA extraction methods in one of the subsamples (e.g. mechanically grinding the samples and using a silica column-based DNA extraction method) is highly recommended. On the other hand, current primers used for feather mites (Dabert et al. 2008) have been found to present several mismatches with some species and to influence the amplification success. Accordingly, future DNA metabarcoding studies are encouraged to focus on designing new degenerated primers that diminish as far as possible the number of mismatches (Elbrecht & Leese 2017).

Host-specificity, transmission dynamics, and trophic generalism of feather mites:

Chapter 7 reveals feather mites as highly host-specific symbionts based on the analysis of the raw, geographic and phylogenetic host-specificity of feather mites at a global scale. In other words, most feather mites inhabit one or some few closely related bird species. In addition, birds bear the same communities of feather mites across their distributional range. **Chapter 4** investigates the transmission dynamics of feather mites. Massive vertical

transmission from parents to offspring at the nest during the breeding season was revealed. Also, evidence of transmission by other means (e.g., phoresis) was not found. Lastly, results suggested that primary selective pressure behind this transmission strategy is the intraspecific competition between mites.

These results support feather mites as highly host-specific and specialist symbionts, in which the main mode of transmission is strongly dependent on their hosts (Mironov & Malyshev, 2002; Doña et al. 2017a). This mode of transmission has been found to be linked to cospeciation in other symbionts (Page, 2006; Clayton et al. 2016), given that factors reproductively isolating host individuals and populations may also isolate their symbiont populations (Clayton et al. 2016). Accordingly, these results have substantial implications to understand the coevolutionary dynamics and diversification of feather mites (as discussed below and in **chapters 6**, **7**, and **8**; Doña et al. 2017a, b, 2018).

Interestingly also, these transmission dynamics are congruent with current evidence on population dynamics of feather mites (i.e. feather mite infrapopulations usually reach the highest abundances before bird's pre-breeding stage, Dubinin, 1951; Pap et al. 2010), supporting that transmission opportunity may be shaping population dynamics of feather mites. Moreover, intraspecific competition was found to influence transmission strategy, and this strategy was also found to be compatible with a maximization of the inclusive fitness of the individuals. However, further research is needed to understand the role of intraspecific competition and inclusive fitness in the diversification of highly specialized and host-specific symbionts (Poulin, 2011).

Chapter 5 shows feather mites as generalist symbionts which feed upon fungi and bacteria present in host feathers. Most importantly, considering these results, the type of relationship of feather mites with birds may be context-dependent (Bronstein, 1994). Thus, feather mites type of interaction may range from mutualism to commensalism (event to parasitism under some unlikely circumstances) according to ecological factors (Blanco et al. 2001; Doña et al. 2018a). These results are highly relevant to understand the coevolutionary dynamics of feather mites but pose a complex selection scenario (e.g., Canestrari et al.

2014). On the one hand, if feather mites were commensalists, coadaptation could not have happened because it imposes unidirectional selection (Clayton et al. 2016). On the contrary, a degree of coadaptation may be expected if feather mites were mutualists (Janzen, 1980; Clayton et al. 2016). Thus, a key point in the understanding of the coevolutionary dynamics of feather mites would be to ascertain whether birds remove (or not) feather mites when preening. In this regard, current evidence supports that birds do not remove feather mites when preening as they do with wing feather lice (Choe & Kim, 1989; Blanco et al. 1997). Indeed, preening has been revealed as the primary selective pressure behind the host-specificity of feather lice (Bush & Clayton, 2006). Further experimental research is needed to reveal the coevolutionary dynamics and the selective landscape of the bird-feather mite host-symbiont system.

The trophic generalism of feather mites is also relevant to understand the dynamics of host-switching of these symbionts (**chapters 6**, **7**, and **8**). Feather mites were presumed to be highly specialized to feed on the uropygial gland oil of their bird hosts (Dubinin, 1951; Proctor, 2003). This oil is highly variable between hosts, and therefore feather mites were expected to be highly specialized to host oil (Jacob & Ziswiler 1982; Proctor, 2003). Results here support, on the contrary, that feather mite mostly rely on fungi and bacteria for diet and that they are generalists regarding the species they feed upon. So, they will not be hampered by diet when switching to a new host. However, resource abundance may be limiting, and thus competition for food resources may play a role in the establishment.

Interspecific competition has been found to be highly relevant for coevolutionary dynamics and diversification processes of other symbionts, such as feather lice (Bush & Malenke, 2008). In feather mites, interspecific competition has been suggested to be behind infrapopulation abundance and spatial preferences (Choe & Kim 1988, 1989, 1991; Mestre et al. 2011; Fernández-González et al. 2015). That is, some species of feather mites coexist with other feather mite species in the same bird but this has consequences for infrapopulation sizes and spatial distribution (Choe & Kim 1988, 1989, 1991; Mestre et al. 2011; Fernández-González et al. 2015). Food resources and plumage microsites have been hypothesized to

be the main factors responsible for this competition (Mestre et al. 2011; Fernández-González et al. 2015).

One of the most remarkable examples of how competition has shaped spatial preferences in feather mites is the different distribution on feathers of *Trouessartia* and *Proctophyllodes* mite species (Proctor, 2003; Mestre et al. 2011). *Trouessartia* mites occupy the dorsal side of feathers (and they present morphological and behavioural adaptations to do so), while *Proctophyllodes* (and all other feather mite genera inhabiting wing flight feathers) occupy the ventral side (Proctor, 2003; Mestre et al. 2011). Competition for food resources was hypothesized to be modulating this coexistence (Fernández-González et al. 2015). Interestingly, in **chapter 5**, an overlap in diet between mite species from these genera was found. Therefore, revealing ongoing competition for food resources between species of these genera, and that current spatial niche partitioning may be "the ghost" of past competition (Fernández-González et al. 2015). Further research is needed to understand the role of interspecific competition and coexistence in feather mites' diversification.

Additionally, while being highly host-specific symbionts mostly inhabiting closely related bird species (**chapter 7**), current patterns of associations of feather mites and birds (**chapter 1,** Jovani et al. in prep.) indicate that hosts coinhabited by closely related mite species are rare. Therefore, suggesting that exclusive competition is taking place (Clayton et al. 2016). Nonetheless, there are some examples of coexistence between closely related mite species, such as the coexistence of *Proctophyllodes motacillae* and *Proctophyllodes macedo* in wagtails (Doña et al. 2016; J. Doña, personal observation). In these cases of coexistence between closely related species, in addition to competing for food resources, mite species are also expected to compete for the best regions of the ventral side of feathers (in contrast to the *Proctophyllodes – Trouessartia* relationship discussed above). These may represent examples of condition-dependent competition (i.e., a type of resource partitioning mediated by variation in environmental conditions; Parsons, 1996; Clayton et al. 2016), as it has been found for coexisting feather lice species of the genus *Columbicola* (Johnson et al. 2009; Malenke et al. 2011). Indeed, feather mites are known to be sensitive to climate

conditions, especially to temperature (Meléndez et al. 2014). However, which factors are behind the coexistence of closely related mite species, whether condition-dependent competition exists and its link to feather mites' diversification merits further study. Interspecific interactions of feather mites with other organisms inhabiting birds, such as feather lice, is also expected. For instance, there is anecdotal evidence supporting feather lice as predators of feather mites (Pérez & Atyeo, 1984). In contrast, feather mites are known to use feather lice egg shells for molt (Pérez & Atyeo, 1984). However, these interspecific interactions have received little attention and need to be further investigated.

The diversification of highly host-specific and specialized symbionts:

Chapter 6 and **7** show strong evidence at a macroevolutionary scale of host-shift speciation as the primary driver of the diversification of feather mites. **Chapter 8** explores the eco-evolutionary scenario of host-shift speciation and shows a more dynamic than anticipated coevolutionary scenario in which ecological filters govern host-shift speciation dynamics.

Chapter 6 examines the cophylogenetic dynamics of feather mites at a fine scale. Cophylogenetic analyses showed little congruence between the phylogenetic tree of birds and mites, far from that predicted by a scenario of strict cospeciation (Fahrenholz, 1913; de Vienne et al. 2013; Clayton et al. 2016). Such extensive signature of host-shift speciation was unexpected for highly specialized and host-specific symbionts and is therefore congruent with concerns on the overestimation of cospeciation stated by de Vienne et al. (2013). Also, they support ecological fitting as a relevant process behind symbiont colonization (Agosta & Klemens, 2008; Agosta et al. 2010).

Overall, these results encourage us to abandon the usual dichotomic view common in symbiont diversification research where the question is whether cospeciation is more relevant than host-shift speciation in a system, by another one focusing on the details of the host-shift speciation diversification history.

Chapter 7 explores the current prevalence of major host-switches and its effects on the diversification of feather mites at a broader scale. Major host-switches were found to be very rare, but overall were needed to explain the origin of at least 21% and 38% of species and genera of feather mites.

In these highly specialized and host-specific symbionts, after a rare major host-switch occurs, gene flow with the donor population is unlikely, and thus they are likely to undergo strong disruptive selection (Agosta & Klemens, 2008; Nyman, 2010; Janz, 2011). **Chapter 7** represents the most extensive study on the relevance of major host-switches for the diversification of symbionts in which transmission is limited (Zietara & Lumme, 2002; Agosta & Klemens, 2008; Agosta et al., 2010; Nyman, 2010; Janz, 2011; Forbes et al., 2017). Notably, this chapter exemplifies how during the diversification history of symbionts, rare processes at an ecological scale may scale up to substantial consequences at an evolutionary scale.

Chapter 8 investigates host-shift speciation at an ecological (microevolutionary) scale. Stragglers were more prevalent than anticipated (a 7.1% of total infrapopulations). Also, a pattern of preferential straggling was found, thus revealing that ecological filters are driving straggling and host-switching.

This study reveals straggling as a massive process, and thus supports that ecological filters are key to understand host-specificity of such specialized symbionts. In this sense, stragglers were found in birds phylogenetically less related than expected by the natural host-range of mite species, but within the range of variation of body mass. This pattern of preferences may be reflecting that to fit ecologically in their hosts, feather mites need to match some host traits which are related with host body mass, e.g., with the inter-barb distance of feather barbules. Also, stragglers were found coinhabiting the same host individual with other mite species more than in natural associations, thus supporting that competition opportunities are real (note that given the degree of specialization of feather mites, they may have been unable to stay at all in a different host). Altogether, preferential straggling to hosts from the same network module suggests that modules are acting as

coevolutionary units, as it has been found in other coevolving systems (Olesen et al. 2007; Bronstein, 2015). This result, jointly with the competition opportunities, suggests that a dynamic arena of coevolution is occurring and that straggling plus interspecific competition are relevant factors (Bush & Malenke, 2008). Time-calibrated phylogenies using markers useful at the population level would help to understand these coevolutionary dynamics.

This coevolutionary scenario is compatible with that predicted by the geographic mosaic of coevolution (Thompson, 2005; Clayton et al. 2016). This should encourage further research studying potential mosaics of selection to verify whether highly specialized and host-specific symbiont systems operate as isolated units of coevolution or instead as a geographic mosaic of coevolution, as found for other less intimate coevolving partners. Finally, the over-prevalence of straggling and host-switching (**chapter 8**) found in feather mites coupled with the preferential straggling found (**chapter 8**) also raise questions on the cases of cospeciation discovered in **chapter 7**. This is because preferential straggling and host-switching may also lead (i.e., as cospeciation does) to congruent phylogenies, i.e., pseudocospeciation (Brooks, 1979; de Vienne et al. 2007). Time-calibrated phylogenies are needed to investigate whether these cases of cospeciation are true cospeciation events and not due to pseudocospeciation (de Vienne et al. 2013). A major problem for time-calibrated phylogenies of feather mites is the lack of fossils, and therefore of calibration points. However, well-dated cospeciation events can be used as calibration points (e.g. Johnson et al. 2018). We are currently using whole genome data to investigate the cospeciation event of feather mites from sister species of azured-winged magpies, and thus to use it later for time-calibrated phylogenies (Zhang et al. 2012).

Synthesis:

This thesis provides resources and molecular tools which allow studying feather mites in a way that was unthinkable before this. Results obtained challenge our previous view on many aspects of the evolutionary ecology of feather mites. This thesis shows feather mites

as highly specialized and host-specific symbionts living on dynamic communities in ecosystems (i.e., hosts) in which they are subject to a plethora of interactions which impact their diversification. In this ecosystem, transmission is limited and intra- and interspecific competition play an essential role in the eco-evolutionary dynamics. Straggling is surprisingly revealed as a prevalent process which allows colonizing new hosts, and ecological fitting govern straggling and host-switching. Main factors behind ecological fitting may be the morphological match between feather mites and birds, and interspecific competition. Also, the more dynamic than expected ecological scenario found here suggests that for highly specialized and host-specific symbionts a geographic mosaic of coevolution may also be expected. Finally, this thesis evidences the needed of a community-level understanding of host ecosystems to understand symbiont diversification adequately.

Conclusions

1. A global dataset of associations between feather mites and birds was compiled. This is the most comprehensive data set available of feather mites and their hosts. Data include 12,036 records of 1,887 feather mite species located on the flight feathers of 2,234 bird species from 147 countries.

2. DNA barcoding and minibarcoding was revealed as a useful molecular tool to identify feather mites, and for the integrative taxonomy of this group of symbionts.

3. DNA metabarcoding allows the correct identification and quantitative estimation of the relative abundance of feather mite species in complex samples.

4. Feather mites' main mode of transmission is vertical. They transmit massively from parents to offspring during the stay in the nest.

5. Vertical transmission magnitude of feather mites is influenced by intraspecific competition.

6. Fungi and potentially bacteria are the main food resources for feather mites.

7. Feather mites are revealed as commensalistic–mutualistic ectosymbionts of birds.

8. Host-shift speciation, rather than cospeciation, is the main driver of symbiont diversification even for highly specialized symbionts with low host-switching potential, such as feather mites.

9. Feather mites are highly host-specific symbionts, whose assemblages do not show geographical structure.

10. Major host switches are very rare events with strong macroevolutionary consequences for feather mite diversification.

11. Straggling and host-switching are highly prevalent phenomena even for highly specialized and host-specific symbionts.

12. Straggling and host-switching set the stage for a geographic mosaic of coevolution where host-symbiont network modules would be the arena of highly dynamic coevolutionary and codiversification processes.

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