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ORIGINAL ARTICLE

Unforeseen biogeographical patterns in a multiple parasite system in Macaronesia

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ABSTRACT

Aim Understanding the reasons why hosts lose, maintain or swap their parasite burden after colonizing new areas has long intrigued island biogeographers. Using molecular markers, we evaluated parasite biogeography of a multiple parasite system of Apicomplexa protozoans (haemosporidian and coccidian parasites) showing different modes of transmission in the spectacled warbler (*Sylvia conspicillata*), a small passerine with recently founded populations in Macaronesia.

Location Twelve oceanic islands of three Macaronesian archipelagos (Madeira, the Canary Islands and Cape Verde) and two continental areas (the Iberian Peninsula and Morocco).

Methods We amplified and sequenced fragments of the parasite mitochondrial genome (cytochrome *b* for haemosporidian and cytochrome *c* oxidase subunit I for coccidian parasites) to determine the prevalence, richness, diversity and associations between groups of pathogens from the continental sources to the Macaronesian islands. We also built a haplotype network for the coccidian parasites to obtain insights on their evolutionary history.

Results We did not find a significant reduction in parasite diversity in the Macaronesian islands compared with the mainland. The prevalence was higher in Macaronesia for both parasite groups than on the mainland, although it was only significant for the haemosporidian parasites. The haplotype network revealed an unexpectedly strong differentiation in the coccidia, with a similar structure to the haplotype topology previously found in the host.

Main conclusions Oceanic islands provide model systems for disentangling geographical origins, colonization pathways, and diversification of avian hosts through analysis of parasites. We show that analysis of coccidians, which closely track the evolutionary history of their avian hosts, may illuminate co-speciation processes in both groups. Our results show that coccidian populations in Macaronesia are highly structured but fail to provide support for the predicted impoverishment of parasite assemblages on islands.

Keywords

Apicomplexa, Haemosporida, host-specificity, island biogeography, island evolution, *Isospora*, Macaronesia, multiple parasite system, parasite biogeography, *Sylvia conspicillata*.

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INTRODUCTION

Within wild animals, much effort has been dedicated to investigating parasite burden in birds because of their potential for pathogen dispersal, with some avian parasites being highly pathogenic to humans, for example avian influenza, West Nile virus, chlamydiosis and cryptosporidiosis (Kurt *et al.*, 2003; Dhama *et al.*, 2008; Abreu-Acosta *et al.*, 2009). Emerging diseases can have a severe impact on bird species and populations (Robinson *et al.*, 2010), a problem that is particularly pronounced for taxa with restricted distributions, such as endemic land bird species inhabiting oceanic islands (van Riper *et al.*, 1986; Carrete *et al.*, 2009). Recent island biogeographical research has increased our knowledge on topics such as patterns of parasite diversity (Ishtiaq *et al.*, 2010; Cornuault *et al.*, 2013), evolution of parasite island syndromes (Pérez-Rodríguez *et al.*, 2013a), the establishment of exotic parasites (Ewen *et al.*, 2012), the influence of offshore winds on haemosporidian prevalence and diversity (Clark & Clegg, 2014), associations of haemosporidian parasites with hosts and vectors (Ishtiaq *et al.*, 2008), and ecological factors governing the distributions of parasites (Spurgin *et al.*, 2012; González-Quevedo *et al.*, 2014). Much of this research has been restricted to vector-transmitted parasites, and involved either insular hosts with distinctive linages or host species with limited geographical ranges. While we have gained insights into the ecology and evolution of blood parasites in oceanic islands, we have only a limited understanding of the patterns and processes characterizing the assembly of island parasite communities in multiple pathogen systems over wide latitudinal gradients entailing significant climatic and environmental variation.

Haemosporidian parasites (Phylum Apicomplexa) have received much attention because they have been involved in the decline and extinction of some 60 endemic Hawaiian bird species (van Riper *et al.*, 1986; Sodhi *et al.*, 2007). The routine task of amplifying parasite sequences from host tissue using the polymerase chain reaction (PCR) has dramatically improved our knowledge of the genetic diversity of these vector-borne protozoans (Bensch *et al.*, 2009). The life cycle of haemosporidian parasites requires two different hosts (one vertebrate and one invertebrate) to be completed. Briefly, there is an initial asexual reproduction developed within erythrocytes and tissues of vertebrate hosts after sporozoites are inoculated. Gametocytes are produced in the vertebrate host, but gametogenesis and fertilization take place in the invertebrate hosts. The zygote is developed as an oocyst, which produces thousands of sporozoites (asexual reproduction). Finally, sporozoites will move to the invertebrate's salivary glands to complete the life cycle (LaPointe *et al.*, 2012). Most haemosporidian parasite studies have been conducted on vertebrate hosts (Santiago-Alarcon *et al.*, 2012). In addition, biogeographical comparisons between parasites with different modes of transmission are currently lacking, and it could difficult to disentangle the causes for haemosporidian distribution.

Coccidian parasites are non-vector-borne gastrointestinal parasites, also included in the Phylum Apicomplexa. This is a diverse group with potential to produce an epizootic in birds, with the occurrence of morbidity and mortality due to coccidiosis (e.g. Schoener *et al.*, 2013). These protozoans can occur in intestinal and extra-intestinal forms, depending on the parasite life-cycle stage. Coccidian transmission is produced via oocysts released in faeces, and these oocysts must sporulate (asexual reproduction) in the environment to be transformed into infective oocysts. Transmission occurs when an uninfected bird takes in the infective oocysts present in the water, food or soil. Once the sporulated oocysts have invaded the new host two new reproduction phases (asexual and sexual) occur to complete the coccidian life cycle (Friend & Franson, 1999). Remarkably, avian coccidia seem to be host-specific, with one or a few lineages per bird species (Schrenzel *et al.*, 2005; Schoener *et al.*, 2013). Despite such host-specificity, the study of genetic characterization, prevalence and biogeographical patterns in coccidian parasites remain under-investigated.

Oceanic island ecosystems provide unique opportunities for the study of past and present distributions of taxa because of their inherent discrete geographical nature, diversity of habitats and climatic conditions (Whittaker & Fernández-Palacios, 2007). This, together with their rich endemic biota, makes island systems ideal environments in which to explore the roles of colonization, diversification, assembly and extinction in parasite communities. Macaronesia is a geologically complex biogeographical region situated in the north-east Atlantic Ocean, composed of five oceanic archipelagos (Azores, Madeira, Selvagens, the Canary Islands and Cape Verde) with 31 volcanic islands supporting a high level of endemic biota. Awareness of evolutionary histories of many bird species has burgeoned in the last two decades (Illera *et al.*, 2012), although the attention dedicated to studying their parasite communities has been limited (e.g. Spurgin *et al.*, 2012; Pérez-Rodríguez *et al.*, 2013a). Nevertheless, the broad latitudinal gradient, spanning temperate (39° N 31° W) and tropical (15° N 23° W) areas, the wide range of distances between islands and the mainland, the high habitat and climatic diversity and the variation in the age of the islands (Illera *et al.*, 2012) provides a rich backdrop for the biogeographical study of host–parasite interactions.

In this study we address the question of what determines the pathogen (haemosporidian and coccidian) diversity and distribution in the spectacled warbler (*Sylvia conspicillata*) in Macaronesia. This bird inhabits temperate to tropical latitudes across geographically disjunct and patchy populations in the Mediterranean Basin, and has colonized three oceanic archipelagos in the Atlantic. In addition, the spectacled warbler populations in Macaronesia have only recently been founded in the last 10,000 years (Illera *et al.*, 2014). Such a model offers an ideal opportunity to test biogeographical hypotheses about variation in prevalence, loss and gain of parasite species, and associations between groups of pathogens, in a recent colonizer. Specifically we evaluate five hypotheses, as follows.

1. Island biogeography theory predicts that continental birds will harbour greater parasite richness than island birds: this is a result of the colonization process, whereby islands retain only a subset of the species from the continental source pool (MacArthur & Wilson, 1967). Consequently, we predict that there will be lower parasite richness and diversity in Macaronesia than on the mainland. In addition, we predict that the most common haplotypes existing on the mainland are more likely to be found on the islands than the rarer ones (Ewen *et al.*, 2012; Pérez-Rodríguez *et al.*, 2013a).

2. Pathogen richness is modulated by host population size. Greater host density should provide the parasite community more opportunities to establish within a host population (Arneberg *et al.*, 1998). Therefore, we predict that we should find more pathogens (in terms of prevalence, richness and diversity) with increasing island size (Spurgin *et al.*, 2012; Pérez-Rodríguez *et al.*, 2013a), which should be related to the abundance of the host (spectacled warblers; see below).

3. Haemosporidian parasites have a complex life cycle and need the presence of dipteran vectors, in which sexual reproduction occurs. However, both hosts (bird and invertebrate) could be independently affected by the distance of an island from the source of colonists, limiting the settlement of the haemosporidian parasites in the most isolated islands. As a result, we predict that insular parasite richness, diversity and prevalence will be more strongly correlated with their proximity to the mainland in haemoparasites than in coccidians (which are not vector-borne parasites). We propose the term 'multiple host handicap hypothesis' to describe the disadvantage of requiring more than one host to complete the parasite life cycle.

4. There is a well-recognized trend of increasing species richness from temperate to tropical areas (e.g. Willig *et al.*, 2003). Nevertheless, the studies tackling variation in latitudinal parasite species richness are scarce and there is no agreement on the generality of this pattern (Poulin, 2007; Poulin *et al.*, 2011). However, it seems that there is a tendency to find higher diversity of protozoa transmitted by arthropods near the tropics (e.g. Nunn *et al.*, 2005; Clark *et al.*, 2014; but see Merino *et al.*, 2008). We therefore predict that there will be higher diversity in the tropics (i.e. Cape Verde) than in temperate archipelagos (i.e. Madeiran and the Canary Islands).

5. Host infection by more than one parasite group is a common pattern in wild animals (Petney & Andrews, 1998). However, information on how parasites interact is often elusive, and consequences on co-infection can provide contradictory results in effects from different studies (Knowles, 2011). Positive associations between groups of pathogens in natural animal populations have been recorded recently (Atkinson *et al.*, 2005; Ezenwa *et al.*, 2010; Spurgin *et al.*, 2012), suggesting that infection with one pathogen could reduce host resistance, favouring infection by others. Therefore, we predict that there will be significant associations between haemosporidian and coccidian parasites at the individual level.

MATERIALS AND METHODS

Sampling

Spectacled warblers were sampled from 14 sites (12 oceanic islands, plus the Iberian Peninsula and Morocco) covering a wide latitudinal gradient of 3580 km. Sampling was carried out at multiple localities within each site (except in North Africa where only one locality was surveyed; Figs 1 & 2), to avoid bias related to intra-site pathogen structure (González-Quevedo *et al.*, 2014). Most birds were caught during 2010 and 2011 (see Illera *et al.*, 2014), with extra samples collected during 2012 and 2013 in La Palma ($n = 7$), the Iberian Peninsula ($n = 22$) and Fuerteventura ($n = 6$). All birds (22–54 per site; Table 1) were trapped using mist nets during the breeding period, and they were kept in clean individual ringing bags until they were ringed. Also, by chance some individuals of blackcaps (*Sylvia atricapilla*) and wild canaries (*Serinus canarius*) were caught in Porto Santo and the Cape Verde islands. Both species were also blood sampled and analysed because of the scarcity of haemosporidian information on those islands. All warblers were also examined for evidence of pox lesions (see Illera *et al.*, 2008). Blood samples were obtained by brachial or jugular venipuncture, preserved in pure ethanol and stored at room temperature. Finally, to detect gastrointestinal parasites we collected all fresh faecal samples produced in the ringing bags and placed them in 800 μL of 2% aqueous solution of potassium dichromate. In order to avoid contamination, when a ringing bag was stained with a faecal sample the bag was not used again until it was cleaned. Faecal samples were stored for 3–7 days at room temperature to facilitate sporulation and then kept in the fridge at 4 °C. All birds were released at the same locations as where they were trapped.

Molecular procedures

Haemosporidian

DNA from blood samples was extracted using Qiagen's DNeasy blood & tissue kit (Qiagen Inc., Hilden, Germany), using the manufacturer's protocol for animal tissue. All birds were sexed using the primers P2 and P8 primers, using the method described in Griffiths *et al.* (1998).

Haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) were screened with a nested PCR that amplifies a 507 base pair (bp) fragment of the mitochondrial cytochrome *b* gene. See Appendix S1 in Supporting Information for further details.

Sequences were edited and aligned by eye using BioEdit 7.2.5 (Hall, 1999) and Chromas Lite 2.1.1 (http://technelysium.com.au/?page_id=13). Sequences were identified as known haplotypes if there was 100% of coincidence with homologous sequences published in the National Centre for Biotechnology Information (NCBI) and MalAvi (Bensch

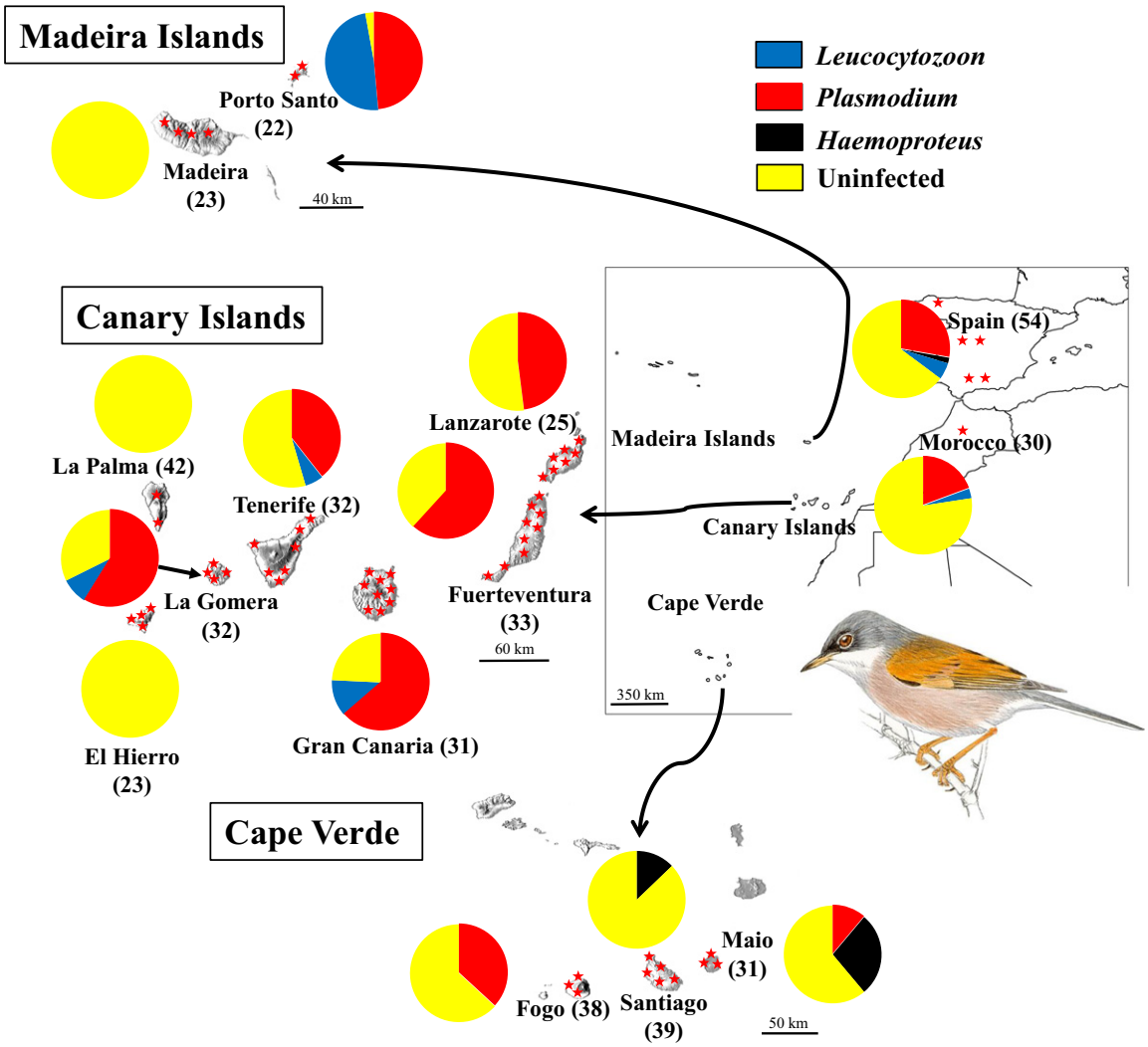

Figure 1 Prevalence (n = 455) of spectacled warbler (*Sylvia conspicillata*) haemosporidians per site is shown with pie charts (sample size in parentheses). The localities where birds were sampled are roughly shown with red stars. Different colours represent each haemosporidian genus (i.e. *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) and uninfected (yellow) birds.

et al., 2009; http://mbio-serv2.mbioekol.lu.se/Malavi/), or new haplotypes if there was at least one bp difference.

Coccidian parasites

To determine coccidian prevalence we first filtered faecal samples through gauze to remove the larger debris. Then oocysts were concentrated and examined using density-gradient flotation with Sheather's sugar solution (SG = 1.30) (Sheather, 1923). DNA was extracted directly from aliquots of positive faecal samples with five or more sporulated oocysts using the Fast Prep (Qbiogene, Illkirch Cedex, France) procedure, after three washing steps with PBS-EDTA to remove potassium dichromate. The DNA released from disrupted oocysts was purified using the Fast DNA ® Spin kit for Soil (Qbiogene, Illkirch Cedex, France) and stored at 4 °C.

From coccidian samples we amplified a 648 bp fragment of the cytochrome *c* oxidase subunit I mitochondrial gene (*COI*), using the primers: Cocci_COI_For (5′-GGTTC AGGTGTTGGTTGGAC-3′) (Ogedengbe *et al.*, 2011), and a new reverse primer designed by us Cocci_COI_AM (5′-CC AAGAGATAATACGAAATGG-3′). See Appendix S1 for further details.

As with the haemosporidian pathogens, samples were sequenced, and either identified as known haplotypes if there was 100% of coincidence with homologous sequences published in the NCBI, or new haplotypes if there was at least one bp difference.

Statistical analyses

We used Chao2, Simpson's and Shannon–Wiener's indices (Magurran, 2004) to estimate α-diversity of both haemosporidian and coccidian pathogens in the 14 sites studied. We performed randomization tests to assess whether significant differences occurred between two sites. Briefly, first the

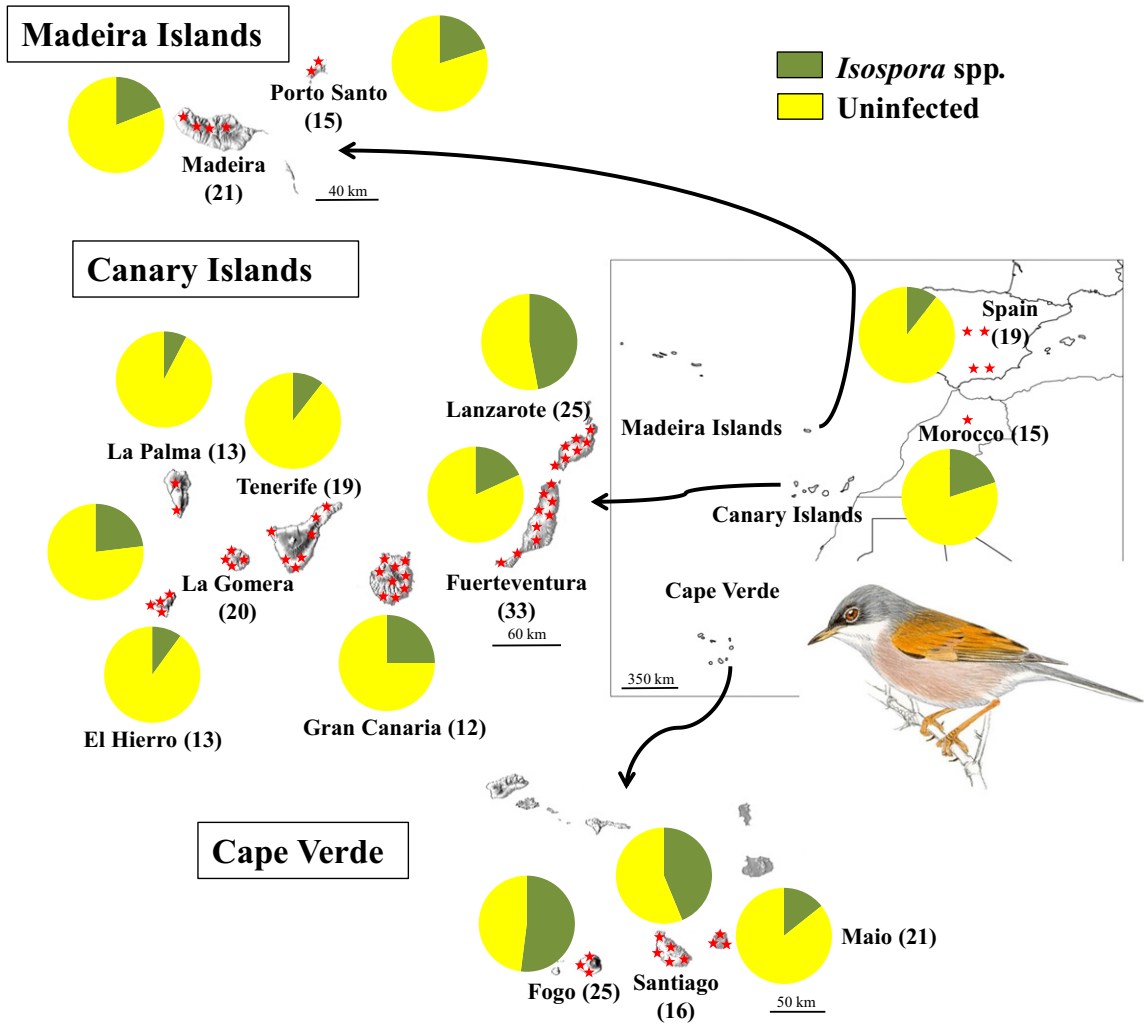

Figure 2 Prevalence (n = 248) of spectacled warbler (*Sylvia conspicillata*) coccidians is shown with pie charts (sample size in parentheses). The localities where birds were sampled are also roughly shown with red stars. Green represents coccidian prevalence and yellow uninfected birds.

diversity difference between samples is obtained using the observed data, then the data are re-sampled 10,000 times to generate a distribution of random differences to compare against the observed values (Solow, 1993). Diversity analyses were performed with the programs SDR 4.1.2 (Seaby & Henderson, 2006) and R 3.1.1 (R Core Team, 2014) with the package FOSSIL (Vavrek, 2011). We performed linear regression analyses using latitude as an explanatory variable and mean diversity as the response variable to test the hypothesis of increasing species diversity from temperate to tropical areas.

We analysed differences in overall prevalence between the mainland and Macaronesia by means of chi-square tests. To test for differences in infection rates (prevalence) between the mainland and the Macaronesian haemosporidian haplotypes we carried out the following analytical approach. First we performed 10,000 bootstrap replicates of birds sampled in the mainland and in Macaronesia using the observed frequencies (sample size of each site is shown in Appendix S2), with the final objective of obtaining expected prevalence values for each haplotype shared. We then performed subsequent Mann–Whitney *U*-tests, for those haplotypes shared between the mainland and Macaronesia, or among archipelagos, to assess whether such differences were significant. Bootstrap and Mann–Whitney *U*-tests analyses were performed in R 3.1.1.

We analysed the effects of island size and isolation (i.e. distance to the nearest continental area) on haplotype richness, diversity and prevalence for both haemosporidian and coccidian parasites (taking into account the distance of the island from the nearest source). We performed multiple regressions where island size, distance to the nearest source, and isolation were the explanatory variables; and richness, diversity and prevalence were the response variables. We used island size as a surrogate measure of host site size because there is a strong association (r = 0.99; $P << 0.01$) between island size and number of 5 km × 5 km squares where the species has been found in the Canary Islands

Table 1 Distribution of haemosporidian haplotypes infecting spectacled warblers (*Sylvia conspicillata*) in Macaronesia and the mainland. *n*, sample size; P, *Plasmodium*; H, *Haemoproteus*; L, *Leucocytozoon*. The lineage name is also given.

Haemosporidian		*Plasmodium*					*Haemoproteus*		*Leucocytozoon*							
	n	P-LK06	P-SC-CV1	P-GRW06	P-SC-IP1	P-GRW11	H-CWT3	H-SC-IP1	L-SC-GC1	L-SC-GC2	L-SC-MO1	L-H027	L-SC-IP1	L-SYAT22	L-SC-PO1	L-SC-TF1	L-SC-PO2
Population																	
Iberia	54	13	0	0	1	1	0	1	1	0	0	1	1	0	0	0	0
Morocco	30	6	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Madeira																	
Porto Santo	22	17	0	0	0	0	0	0	9	4	0	0	0	2	1	0	1
Madeira	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Canary Islands																	
Fuerteventura	33	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lanzarote	25	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gran Canaria	31	21	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0
Tenerife	32	13	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
La Gomera	32	20	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
La Palma	42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
El Hierro	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cape Verde																	
Maio	31	0	1	1	0	0	5	0	0	0	0	0	0	0	0	0	0
Santiago	39	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
Fogo	38	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(Trujillo, 2007). Despite the fact that there is no information on warbler abundance per square, we feel confident in the validity of this approach because squares can be roughly considered similar in habitat quality (J.C.I., pers. obs.). Geographical distances were estimated using the approach of Illera *et al.* (2007) and island size information was obtained from Illera *et al.* (2014). All variables were arcsine- (proportional variables) or log-transformed. Finally, a binary logistic regression was carried out to test for associations among the following pathogens: *Plasmodium*/*Haemoproteus* (they were grouped because of the low number of warblers infected with *Haemoproteus* and their close phylogenetic relationships), *Leucocytozoon* and coccidians. We used only individuals screened for all parasites. We ran separate models for each pathogen, with presence/absence infection as the response variable and island, sex (as a confounding variable), and the remaining pathogens as explanatory variables. Regression analyses were performed using program SPSS version 19.0 (SPSS, Inc., Armonk, NY, USA).

Because avian coccidian parasites are considered to be host-specific, we built a statistical parsimony network (Templeton *et al.*, 1992) with *COI* to analyse their evolutionary history. We used the program TCS 1.21, with the maximum number of differences among haplotypes being estimated with a parsimony connection limit of 95% (Clement *et al.*, 2000).

RESULTS

A total of 455 spectacled warblers were caught across the 14 sites studied. In addition, two and eight blackcaps were trapped in Porto Santo and the Cape Verde islands, respectively, and three wild canaries in Porto Santo (haplotypes found are shown in Appendix S3). We found no evidence for lesions compatible with the presence of avian pox in any individual; therefore, all analyses were performed for haemosporidian and coccidian parasites.

Haemosporidian infection

We screened a total of 455 spectacled warblers for haemosporidian infection and PCR results were consistent between duplicated PCRs (99% of coincidence). We did not find evidence of *Plasmodium* and *Haemoproteus* co-infections. Haemosporidian prevalence of infected sites ranged from 13% to 95% (Fig. 1). Birds from three islands (El Hierro, La Palma and Madeira) were free of haemosporidian infection. At the archipelago level the highest prevalence (only islands with pathogen presence) was in the Madeiran archipelago (95%), followed by the Canary Islands (53%), with the lowest prevalence in Cape Verde (24%). The continental areas showed moderate prevalence levels (35% and 20% for the Iberian Peninsula and Morocco, respectively). Overall, Macaronesia showed significantly higher prevalence values than the mainland ($\chi^2 = 9.41$; $P < 0.01$). The dominant genus was *Plasmodium* (76%) in both continental and archipelagos, except in

Santiago and Maio (Cape Verde) where *Haemoproteus* was the dominant genus, and in Porto Santo (Madeira archipelago) where *Leucocytozoon* was the most abundant haemosporidian (Table 1, Fig. 1). *Plasmodium* was present in all three archipelagos and the two continental areas. However, *Haemoproteus* was absent from Madeira and the Canary Islands, while *Leucocytozoon* was not found in Cape Verde (Table 1, Fig. 1).

The genetic characterization of haemosporidians in the spectacled warblers revealed 16 haplotypes: nine *Leucocytozoon*, five *Plasmodium* and two *Haemoproteus* (Table 1). New sequences are available in the NCBI with the accession numbers KP688295–KP688305 (Appendix S3). Most haplotypes (two *Plasmodium*, one *Haemoproteus* and nine *Leucocytozoon*) were either undescribed ($n = 7$) or were identified in 2014 ($n = 4$). However, the most common haemosporidian (*Plasmodium*-LK06, Table 1) has been detected in two other avian hosts, one of which, Berthelot's pipit (*Anthus berthelotii*), is endemic and widespread in Macaronesia (Illera *et al.*, 2008; Spurgin *et al.*, 2012). The unique *Haemoproteus* haplotype retrieved in Macaronesia for the spectacled warbler was a sequence found in Cape Verde. This haplotype was previously detected in *Sylvia communis* in the Iberian Peninsula (see Appendix S3).

Simpson's and Shannon–Wiener's indices showed the highest diversity values in the Madeira archipelago, followed by the mainland and Cape Verde, with the lowest levels in the Canary Islands (Table 2). Randomization tests showed that such diversity differences were not significant ($P > 0.05$) for either index, for all pairwise comparisons involving Madeira, Cape Verde and the continental areas. However, we found significant differences ($P < 0.05$) in both indices in all pairwise comparisons involving the Canary Islands and the remaining sites (i.e. the Canary Islands showed significantly lower diversity compared with other sites). Chao2 diversity index was not significantly different between Macaronesia and the mainland ($Z = -0.004$, $P = 0.99$). Nevertheless, randomization tests showed significant diversity differences among the Macaronesian archipelagos ($P << 0.01$; Table 2). We did not find significant effects of latitude on haemosporidian parasite diversity (Shannon–Wiener, $R^2 = 0.16$, $P = 0.29$; Simpson, $R^2 = 0.33$, $P = 0.39$; Chao2, $R^2 = 0.07$, $P = 0.48$).

We did not find significant effects of island size or island isolation on haemosporidian parasite richness ($R^2 < 0.15$, $P > 0.50$), prevalence ($R^2 < 0.19$, $P > 0.40$), and Shannon–Wiener and Chao2 diversity indexes ($R^2 < 0.44$, $P > 0.08$), but we found a significant effect of island size on Simpson's diversity index ($R^2 = 0.72$, $P = 0.02$).

Only two haplotypes were shared between the mainland and the oceanic islands, albeit with different probabilities of occurrence (Table 1). The most common haemosporidian haplotype (P-LK06) was found in all three Macaronesian archipelagos and the two continental areas; however, it was significantly more abundant in Macaronesia than in the mainland ($U = 2670942$; $P << 0.001$; Appendix S2). Again, the same result was obtained with the other shared haplotype

Table 2 Alpha-diversity values for haemosporidian and coccidian parasites infecting spectacled warblers (*Sylvia conspicillata*) in Macaronesia and the mainland. Indices per site, grouped continental areas (Iberian Peninsula plus Morocco), grouped islands (i.e. Macaronesia) and grouped islands within archipelago are shown. *H*, Shannon–Wiener index; *D*, Simpson's *D* index; Chao2, Chao2 index. Upper and lower 95% confidence intervals obtained with a standard bootstrap are also shown in parentheses. Significant differences among archipelagos, and between Macaronesia and the continent (established with randomization tests) are marked in bold and highlighted in grey.

	$H_{haemosporidian}$	$D_{haemosporidian}$	$Chao2_{haemosporidian}$	$H_{coccidia}$	$D_{coccidia}$	$Chao2_{coccidia}$
Continent	1.11 (0.43–1.41)	1.90 (1.28–3.22)	37.00 (5.00–38.00)	0.64 (0–0.64)	3.00 (1.00–3.00)	2.50 (1.00–2.50)
Iberian Peninsula	1.19 (0.41–1.51)	2.19 (1.40–4.07)	29.00 (4.00–30.00)	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Morocco	0.41 (0.00-0.68)	1.40 (1.00–2.33)	2.00 (2.00–3.00)	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Macaronesia	0.97 (0.73–1.14)	1.71 (1.47–2.03)	23.50 (7.00–26.00)	2.11 (1.36–2.21)	6.12 (2.75–13.04)	**63.00** (7.50–73.50)
Madeira	1.32 (0.89–1.52)	3.13 (2.10–4.19)	**8.00** (5.00–10.00)	**0**	**1.00** (1.00–1.00)	**4.00** (4.00–4.00)
Madeira	n.a.	n.a.	n.a.	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Porto Santo	1.32 (0.89–1.52)	3.13 (2.10–4.19)	8.00 (5.00–10.00)	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Canary Islands	**0.39** (0.20–0.57)	**1.21** (1.09–1.39)	**4.50** (3.00–6.00)	1.91 (1.07–1.98)	9.43 (2.75–13.2)	**26.00** (4.00–29.00)
Fuerteventura	0	1.00 (1.00–1.00)	2.00 (2.00–2.00)	0.69 (0–0.69)	2.00 (2.00–2.00)	3.00 (1.00–3.00)
Lanzarote	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.61 (0.50–1.61)	5.00 (1.67–10.00)	15.00 (2.00–15.00)
Gran Canaria	0.53 (0.17–0.78)	1.41 (1.09–1.97)	3.00 (3.00–5.00)	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Tenerife	0.48 (0.00–0.86)	1.35 (1.00–2.14)	8.00 (5.00–10.00)	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
La Gomera	0.39 (0.00–0.57)	1.31 (1.00–1.73)	2.00 (2.00–3.00)	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
La Palma	n.a.	n.a.	n.a.	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
El Hierro	n.a.	n.a.	n.a.	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Cape Verde	0.95 (0.63–1.15)	2.39 (1.75–2.90)	**5.00** (3.00–6.00)	1.68 (0.85–1.73)	9.00 (2.25–12.00)	**14.00** (3.00–14.00)
Maio	0.80 (0–1.08)	2.10 (1.00–4.20)	2.00 (3.00–5.00)	1.10 (0–1.10)	3.00 (1.00–3.00)	6.00 (1.00–6.00)
Santiago	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.10 (0–1.10)	3.00 (1.00–3.00)	6.00 (1.00–6.00)
Fogo	0	1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.64 (0–0.64)	3.00 (1.00–3.00)	2.50 (1.00–2.50)

n.a., not applicable.

(L-SC-GC1) between the mainland and the oceanic islands ($U = 11014505$, $P << 0.0001$; Appendix S2). Seven haplotypes were only found in the mainland, and eight were restricted to Macaronesia, albeit with low prevalence values in both cases (Table 1, Appendix S2).

Coccidian infection

We screened a total of 248 warblers for coccidian infection. All positives detected by microscopy corresponded to the genus *Isospora*. Coccidian prevalence ranged from 7.69% to 52% (Fig. 2). Prevalence values were higher in Macaronesia (27.57%) than on the mainland (14.7%), although this difference was not significant ($\chi^2 = 1.90$; $P > 0.05$). In contrast to haemosporidian pathogens, the coccidian parasites were present in all 14 sites studied (Fig. 2). The highest prevalence was found in the Cape Verde islands (47% on average). The remaining sites, except Lanzarote, exhibited prevalence levels lower than 24% (Fig. 2). We extracted DNA from 49 samples with five or more sporulated oocysts. However, from these we were only able to obtain good sequences in 28 coccidian positives, which yielded 15 different haplotypes, all of which were new (i.e. unrecorded in the NCBI). Sequences are available in the NCBI with the accession numbers KP688306–KP688320 (Appendix S3). Coccidian α-diversity was higher in Macaronesia than on the mainland (Table 2). Differences were not significant, except with the Chao2 diversity index, where randomization tests provide evidence that they harbour different parasite diversities ($Z = -25.3$, $P << 0.01$). However, as a result of the limited number of sequences obtained per site (especially in the mainland), we were unable to perform further analyses between observed and expected parasites as we did with the haemosporidian pathogens.

We did not find significant effects of latitude on coccidian parasites (Shannon–Wiener, $R^2 = 0.02$, $P = 0.72$; Simpson, $R^2 = 0.07$, $P = 0.49$, Chao2, $R^2 = 0.01$, $P = 0.78$). Furthermore, we did not find significant effects of island size on coccidian parasite richness ($R^2 = 0.46$, $P = 0.06$), prevalence ($R^2 = 0.03$, $P = 0.85$) and diversity ($R^2 < 0.36$, $P > 0.13$, for the three diversity indexes). We did not find significant effects of island isolation on prevalence and diversity of coccidians ($R^2 < 0.43$, $P > 0.09$), but we found a significant effect of the distance of the island from the nearest source on coccidian richness ($R^2 = 0.51$, $P = 0.04$).

The coccidian haplotype network produced 32 parsimony informative sites, and revealed a common haplotype represented by sequences from the three Macaronesian archipelagos sampled (i.e. Madeira, the Canary Islands and Cape Verde), which was closely connected with haplotypes from Lanzarote. Interestingly, Lanzarote is the island with the most unique haplotypes (five), and also appears to be the link connecting the remaining haplotypes (Fig. 3). The haplotype network shows an ongoing genetic divergence from Lanzarote towards Fuerteventura and Gran Canaria, and from these islands to the Cape Verde archipelago (Fig. 3).

Multi-parasite analyses

Logistic regressions showed different infection patterns among parasite communities. Coccidian infection was not associated with the presence of haemosporidian species and was not related to island or sex (Table 3). However, a different pattern emerged with the haemosporidians. *Leucocytozoon* had a significant effect on *Plasmodium/Haemoproteus* infection, and *Plasmodium/Haemoproteus* on *Leucocytozoon* infection. In addition, there was a significant effect of sex on *Plasmodium/Haemoproteus* infection (52.7% of females were positive for the pathogen compared with 30% in males), and island identity on *Leucocytozoon*, largely reflecting the high prevalence in Porto Santo (Table 3).

DISCUSSION

The study of patterns and processes involved in the colonization, diversification and extinction in oceanic biotas has substantially contributed to our understanding of the causes promoting species diversity (Losos & Ricklefs, 2010). We evaluated both widely recognized concepts and theories in island biogeography and recent insights obtained in the study of island parasite biogeography, within a multiple pathogen system in recently colonized oceanic islands. Based on classical biogeographical predictions and recent studies we expected to find an impoverished island haemosporidian assemblage with lower richness and diversity values in Macaronesia (Whittaker & Fernández-Palacios, 2007; Pérez-Rodríguez *et al.*, 2013a). However, our results suggest that there are a similar number of haemosporidian haplotypes on the mainland ($n = 9$) as in Macaronesia ($n = 10$), with no significant differences in terms of α-diversity values. The same result was found for the coccidian parasites, although the low number of sequences obtained limits how much we can infer from this group. We also did not find evidence of a latitudinal diversity gradient from temperate zones to the tropics (cf. Willig *et al.*, 2003). Indeed, the highest haemosporidian diversity value was detected in Madeira, the most temperate archipelago, and the lowest in the Canary Islands. We also failed to find such a latitudinal diversity gradient in the coccidian parasites, but again we need to be cautious about interpreting this because of the low number of sequences retrieved. Other similar exceptions have been found, including oribatid mites (Maraun *et al.*, 2007), avian helminth parasites (Poulin, 2010), and avian haemosporidian (Merino *et al.*, 2008). This result cannot be explained by the higher number of avian and invertebrate host species inhabiting Madeira (Appendix S1). Therefore, it seems likely that other geographical, biotic or human-mediated factors could be behind this unexpected result.

The most common haemosporidian on the mainland (P-LK06) was also the most abundant pathogen in Macaronesia, which was an expected pattern (Pérez-Rodríguez *et al.*, 2013a). However, it was not the case for the second most common haemosporidian in Macaronesia (L-SC-GC1), which was only found in one bird from the mainland

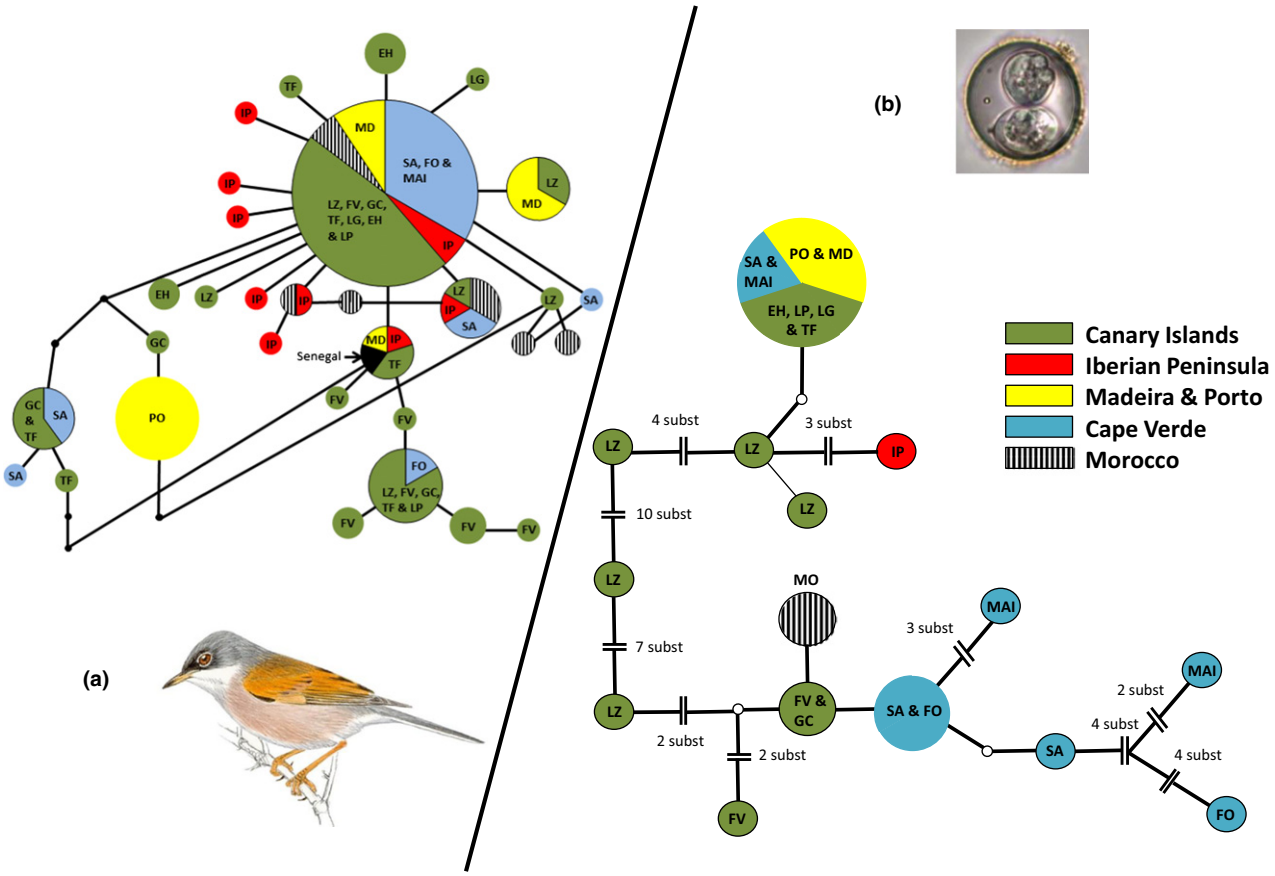

Figure 3 Statistical parsimony network of (a) the spectacled warbler (*Sylvia conspicillata*) based on the cytochrome *b* gene (after Illera *et al.*, 2014); and (b) coccidians infecting the spectacled warbler based on the cytochrome *c* oxidase subunit I gene (*COI*). Open small circles represent one-step mutation edge. Numbers of mutations greater than one are marked with numbers. The size of coloured circles is proportional to the number of sequences per haplotype obtained. Acronyms represent the geographical position of each haplotype: SA, Santiago; MAI, Maio; EH, El Hierro; LP, La Palma; LG, La Gomera; TF, Tenerife; LZ, Lanzarote; FV, Fuerteventura; GC, Gran Canaria; FO, Fogo; MO, Morocco; IP, Iberian Peninsula; MD, Madeira; PO, Porto Santo.

(Table 1). Notably, few warblers were found to be infected with *Haemoproteus* in Macaronesia (Fig. 1), which could be related with the type of habitat (dry sparse scrub) this species inhabits (Pérez-Rodríguez *et al.*, 2013b). Also, the low number ($n = 2$) of haemosporidian species shared between the continent and the islands is striking. This result could be explained by the extremely low prevalence found in all haemosporidian haplotypes (except with P-LK06) on the mainland (i.e. most haplotypes were detected only once). Interestingly, of the 16 haemosporidian sequences found in the spectacled warbler, seven were new haplotypes, not discovered in any other bird host so far and, from these, three were only found on the mainland and four in Macaronesia (Table 1). Because most of the new haemosporidians detected were found only once, despite the high number (> 450) of birds screened, such host specificity could also explain the lack of suitability of the spectacled warbler as a competent host for such haemosporidian species (Valkiūnas *et al.*, 2009). Regrettably, the number of bird hosts screened in Macaronesia and in the close continental areas is very limited, and we cannot confirm or say whether these unique haemosporidian haplotypes are also infecting other bird hosts and with higher prevalence. However, some lines of evidence suggest that this apparent specificity could be a consequence of insufficient sampling effort in other avian hosts. For example, the linage SYAT22 found in two spectacled warblers in Porto Santo (this study) was originally detected in continental blackcaps, but has also been observed in Berthelot's pipit in Porto Santo (Spurgin *et al.*, 2012) and, more recently, in the Canarian blackcaps (Pérez-Rodríguez *et al.*, 2013a). In addition, the originally unique *Leucocytozoon* haplotypes L-SC-GC1 and L-SC-GC2 found in this study in Porto Santo and the Canary Islands have been detected in one blackcap and two wild canaries in Porto Santo (this study, Appendix S3), and recently on the African mainland too (http://mbio-serv2.mbioekol.lu.se/Malavi/index.html; last accessed May 2015). Overall, it appears that sampling additional bird species would increase the chance of detecting these apparently unique haemosporidians in other avian hosts.

Prevalence of haemosporidian haplotypes was significantly higher in Macaronesia than on the mainland (Appendix S2),

Table 3 Logistic regressions showing the effect of haemosporidian (*Leucocytozoon* and *Plasmodium/Haemoproteus*) and coccidian (*Isospora*) pathogens on the probability of infection in the spectacled warbler (*Sylvia conspicillata*) controlling for sex and site (14 sites and 248 individuals analysed). Significant values are highlighted in bold. *B* = coefficient of the logistic regression.

	B	Wald	d.f.	Significance
Coccidia				
Site	−0.05	1.55	1	0.21
Sex	0.27	0.37	1	0.54
Plasmodium	−0.01	0.001	1	0.98
Leucocytozoon	0.44	0.54	1	0.46
74.2% of cases were classified correctly by the model				
Plasmodium				
Site	−0.06	2.48	1	0.11
Sex	−0.94	6.18	1	**0.01**
Coccidia	−0.02	0.01	1	0.96
Leucocytozoon	2.11	11.74	1	< **0.01**
69.8% of cases were classified correctly by the model				
Leucocytozoon				
Site	0.23	8.81	1	**0.01**
Sex	0.12	0.03	1	0.87
Plasmodium	−0.60	0.95	1	< **0.01**
Coccidia	−2.13	11.71	1	0.33
93.5% of cases were classified correctly by the model				

a finding contrary to previous studies (Hellgren *et al.*, 2011; Pérez-Rodríguez *et al.*, 2013a). Moreover, we did not find significant relationships between parasite richness, prevalence and diversity with island size and distance to the mainland (an exception was found with the Simpson index and the island size). These results conflict with our multiple host handicap hypothesis (i.e. stronger associations between parasite richness, diversity and prevalence with distance to the mainland in haemosporidian than in coccidian parasites). In fact, we found a significant effect of the distance of the island to the nearest source on coccidian richness. Also, our findings contrast with recent findings obtained with haemosporidian parasites in the Berthelot's pipit (Spurgin *et al.*, 2012) and the blackcaps (Pérez-Rodríguez *et al.*, 2013a) in Macaronesia, where smaller and isolated islands harbour fewer haemosporidian. Explanations for this unexpected pattern remain elusive but our findings highlight the complex relationships that can occur in a system where two different hosts (one vertebrate and one invertebrate) are needed to complete the parasite life cycle. Thus, we cannot reject the idea that either few dipteran vectors are able to transmit the whole haemosporidian community, or that conditions for transmissions vary across islands, which would limit much of the pathogen geographical distribution (Santiago-Alarcon *et al.*, 2012). Studies on specificity of dipteran vectors transmitting haemosporidian lineages, including physiological and behavioural adaptations, and longevity are now needed, along with climatic information that may affect the pathogen development (especially temperature and humidity; Sehgal *et al.*, 2011), to understand the reasons behind the unforeseen biogeographical patterns found here.

Simultaneous infection by multiple parasites is common in wild animals, although the significance of interactions, and the consequences for their hosts are rarely evaluated in non-model organisms (Pedersen & Fenton, 2007). Here we studied two different types of protozoa (haemosporidian and coccidian) infecting the spectacled warbler to infer the putative associations established between them. We found no evidence for a significant association between haemosporidian and coccidian parasites at the individual level, suggesting that competitive interactions between pathogens were absent or very weak. This could be for two reasons: (1) these protozoans do not share an infection site in the bird (haemosporidian are blood parasites and coccidian are gastrointestinal parasites); and (2) they are transmitted by different mechanisms (vector-borne in haemosporidians, direct ingestion of infective oocysts in coccidians). However, such a significant relationship was detected within the haemosporidian genera analysed (*Plasmodium/Haemoproteus* and *Leucocytozoon*), suggesting that the expected local and dynamic competition for shared host resources was not enough to segregate these two genera. Alternatively, such a result could be explained if the infection by one genus makes warblers more susceptible to the other because of immunosuppressive effects (Spurgin *et al.*, 2012). Common-garden experiments that evaluate the importance of parasite identity, along with order and timing of infection on responses of the host immune system are now needed, to understand the reasons behind the interactions among parasites detected here (Knowles, 2011).

The genetic characterization of parasites provides insights into host-range, richness and diversification that may be crucial to understanding the limits of their transmission and co-speciation. Most of the coccidian parasites are obligate intracellular parasites and presumably many of them are either species or genus specific (Schrenzel *et al.*, 2005; Schoener *et al.*, 2013). However, despite such host-specificity there is a surprising lack of studies testing diversification and co-divergence between the coccidian and their hosts. To our knowledge this is the first study tackling this question in an oceanic system, including their putative source of colonizers. Our results have made two things clear: (1) coccidian parasites are more widely distributed in Macaronesia with haemosporidians more restrictive, slightly supporting the idea of a multiple host handicap in the haemosporidian assemblage; and (2) the haplotype parsimonious network has revealed a remarkable and unforeseen strong diversification process ongoing in the coccidia parasite assemblage, with only three shared haplotypes and, importantly, with a similar structure (albeit better resolved) than the haplotype topology found in the spectacled warbler (Illera *et al.*, 2014). Indeed, the haplotype network strongly suggests a double wave of colonization of the Cape Verde archipelago, with the Canary Islands being the origin for at least one of them (Fig. 3). Such double colonization (and the southward expansion) was hypothesized

to occur with the spectacled warbler based on the number of haplotypes shared by the Cape Verde islands with the Canary Islands, and with the Canary Islands and other populations (Fig. 3), together with the two clusters identified with the microsatellite data (Illera *et al.*, 2014). The Madeiran Islands appear to have been colonized recently from the central or western Canary Islands, based on the common haplotype shared by Madeira and Porto Santo (Madeira), and Tenerife, La Gomera, La Palma and El Hierro (Canary Islands). Colonization origins from south to north is not the most common colonization pattern observed in the Macaronesian archipelagos (Illera *et al.*, 2012), but such a pattern has been previously found in Berthelot's pipit (Illera *et al.*, 2007; Spurgin *et al.*, 2014), another passerine inhabiting open semi-arid habitats in Macaronesia. Results also provide strong evidence of an ongoing differentiation process in the Cape Verde coccidians, which probably colonized from Gran Canaria or Fuerteventura (Fig. 3). Congruent phylogeographical patterns between bird hosts and their ectoparasites have been previously provided for feather lice and mites (e.g. Štefka *et al.*, 2011; Koop *et al.*, 2014), although to the best of our knowledge this is the first time this has been documented in endoparasites. However, although the colonization pattern found is very promising, it should be interpreted with caution because of the limited sample size.

Overall, this study has revealed that processes of colonization and diversification in a multiple parasite system, with different transmission modes, are complex and partially contrary to classical predictions of island biogeography. The haemosporidian community was dominated by few haplotypes and, importantly, it was not impoverished after the colonization of Macaronesia. Intriguingly, the *Sylvia* system provides the opportunity to further investigate co-speciation processes in birds and coccidians because these parasites closely track the evolutionary history of their hosts. Furthermore, they may be invaluable to disentangle geographical origins, pathways of colonization, and diversification of their avian hosts in those cases involving complex colonization histories because coccidian populations are highly structured.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Details of the molecular procedures used and the number of potential hosts for haemosporidians in Macaronesia.

Appendix S2 Expected prevalence of haemosporidian haplotypes.

Appendix S3 Haemosporidian and coccidian (*Isospora* spp.) haplotypes found.

BIOSKETCH

Juan Carlos Illera is interested in studying both ecological and historical processes shaping bird distributions and patterns of genetic variation within and among species inhabiting oceanic islands that are the result of colonization, adaptation and diversification.

Author contributions: J.C.I. obtained funding for and set up the study, supervised the study and performed the fieldwork. J.C.I., A.F-A. and P.F. performed the molecular work. J.C.I. and C.N.H-F. performed the analyses. J.C.I. drafted the manuscript and all authors approved the final version of the manuscript.

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