Divergence between the COI-5P gene regions in the Iberian and European lineages of the Eurasian Green Woodpecker *Picus viridis*

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In terms of morphology, the Iberian lineage *Picus viridis sharpei* of the Eurasian Green Woodpecker has long been known to be significantly different from the nominate subspecies *Picus viridis viridis*. Various mitochondrial DNA-based studies of this species have highlighted the possibility of elevating the Iberian lineage to species level (*Picus sharpei*), a status that has been adopted by certain taxonomies. Recent research has shown that there is reduced gene flow between these two lineages in the areas where they coincide, thereby providing further support for their treatment as separate biological species. In this study we investigated whether or not the divergence in the COI-5P region, a mtDNA marker widely used for species diagnosis, between these taxa fits the divergence rate expected between species. We used the Generalized Mixed Yule-Coalescent (GMYC) method for species delimitation to investigate species limits within this group. Our results revealed great divergence (2.04–2.61%) between the studied mtDNA regions of these two lineages; furthermore, the GMYC analysis consistently separated the Iberian lineage into two separate species. These findings match what is expected to occur between different species. Thus, taking into account these results, along with those of previous studies, we support the specific separation of the Iberian Green Woodpecker.

Key words: Iberian Green Woodpecker, *Picus viridis sharpei*, taxonomy, COI, GMYC.

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Since the turn of the century the popularization of molecular systematics in avian taxonomy has led to a reassessment of many bird taxa, an impact that can best be judged by the ever-rising number of bird species on avian checklists (del Hoyo & Collar 2014, Gill & Donsker 2017). Of the hundreds of species incorporated into checklists over the past two decades, very few have been previously unknown taxa since the main part have been the result of molecular analysis leading to the description of cryptic species or the split of distinct subspecies (Gill & Donsker 2017). Despite elucidating the phylogenetic relationships of a huge number of taxa, the use of molecular techniques has created a new layer of complexity when delimiting taxa that still remains to be fully addressed (Ritchie *et al.* 2018).

In molecular systematics, the mtDNA gene cytochrome oxidase subunit 1 (COI/COI) has proved to be an excellent tool for species diagnosis as it varies substantially between species but not between subspecies belonging to the same species. It has therefore been proposed as the basis of a universal DNA barcode database for identifying animal species (Hebert *et al.* 2003,

2004; Kerr *et al.* 2007). Likewise, species whose COI barcodes show deeply diverging lineages may warrant taxonomic revaluation (Barreira *et al.* 2016). An analysis of a 695-bp region of the COI-5P region of this gene in 260 North American bird species has shown that most interspecific differences exceed 1.25%, while intraspecific differences are mostly inferior to this figure, thereby suggesting that it could be a possible threshold for species delimitation (Hebert *et al.* 2004, Kerr *et al.* 2007). However, there may be a exceptions in which interspecific distances lie below this value (e.g. several *Larus* species) or, conversely, a strikingly deep divergence of up to 7% between a species' least-related populations, as in the Solitary Sandpiper *Tringa solitaria* and Marsh Wren *Cisthorus palustris* (Hebert *et al.* 2004, Kerr *et al.* 2007). Due to such exceptions, it has been argued that the unilateral use of these thresholds for species delimitation is not advisable; rather, it should be used to flag population splits for further investigation, and that it should be taken into account along with other phylogenetic and morphological/biological studies (Baker *et al.* 2009).

Although DNA barcodes are available for nearly half of the world's bird species, there are still gaps in the coverage of certain well-known taxa including the Iberian lineage of the Eurasian Green Woodpecker *Picus viridis* (Linnaeus 1758). This woodpecker has traditionally been divided into five subspecies (Winkler & Christie 2017). The eastern *P. viridis karelini* and *P. viridis innominatus* (Figure 1) are unanimously considered

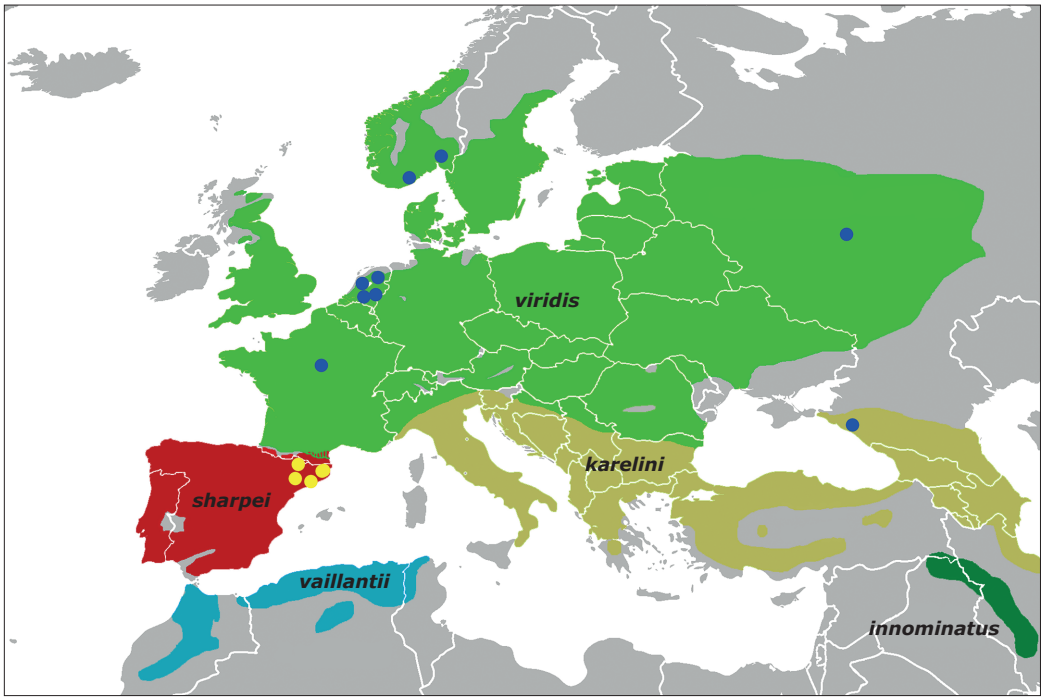


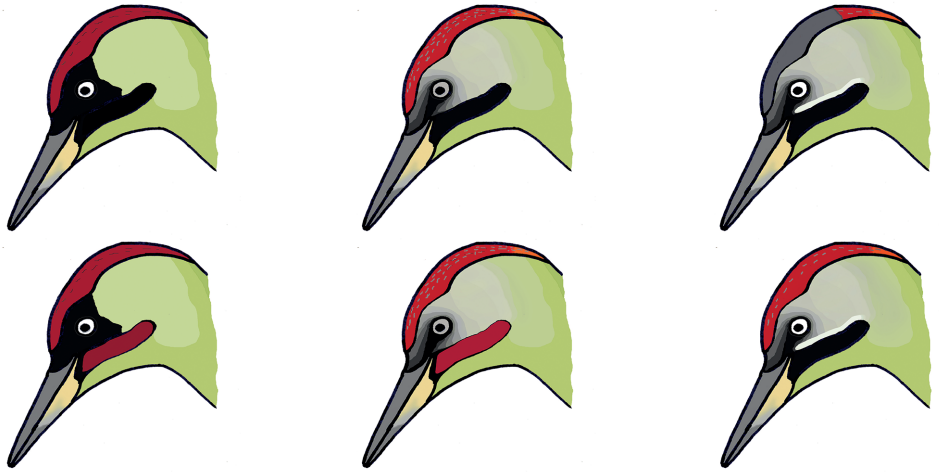
Figure 1. Distribution of the members of the European Green Woodpecker *Picus viridis viridis* species complex (based on Winkler & Christie 2017): ssp. *viridis* (bright green), ssp. *karelini* (yellow-green), and ssp. *innominatus* (dark green). Iberian Green Woodpecker *P. viridis sharpei* lineage (red); and Maghreb Green Woodpecker *P. vaillantii* (blue). Dots mark the origin of the sequences included in the COI-5P regions of the studied taxa: in yellow, the *sharpei* lineage sequences obtained during this study; in blue, the *viridis* lineage sequences obtained from GenBank.
Distribució dels membres del complex del picot verd (basat en Winkler & Christie 2017): ssp. viridis *(verd viu), ssp.* karelini *(verd clar) i ssp.* innominatus *(verd fosc) del picot verd – llinatge* Picus viridis viridis*; picot verd ibèric – llinatge* P. viridis sharpei *(vermell); i picot verd del Magreb –* P. vaillantii *(blau). Els punts marquen l'origen geogràfic de les seqüències incloses en la comparació de la regió COI-5P dels tàxons estudiats: en groc, aquelles pertanyents al llinatge ibèric i obtingudes durant aquest estudi; en blau, les pertanyents al llinatge europeu i obtingudes a partir de GenBank.*

to be conspecific with the nominate subspecies, distributed throughout much of western Europe north of the Pyrenees (Figure 1), due to their very similar morphology that mostly differs in terms of slight variation in the green of their plumages (Winkler & Christie 2017).

By contrast, morphological and ecological differences in the two remaining Green Woodpecker lineages have led to their proposal as separate species. Despite being traditionally treated as subspecies of *Picus viridis,* the specific status of the morphologically distinct Maghreb Green Woodpecker *P. vaillantii* (Malherbe 1847) from NW Africa (Figure 1) is now accepted by all major taxonomic authorities (Dickinson & Remsen 2013, del Hoyo & Collar 2014, Gill & Donsker 2017). However, the status of the Iberian Green Woodpecker, *P. viridis sharpei* lineage (*sensu* Pons *et al.* 2019), is less clear. Despite being treated until recently as a subspecies of the Eurasian Green Woodpecker due to widespread introgression in southern France, morphological differences and genetic divergence in the Iberian Green Woodpecker have led to it be split from *Picus viridis*. This lineage is now considered as a separate species (*P. sharpei*, H. Saunders 1872) by most taxonomic authorities (del Hoyo & Collar 2014, BirdLife International 2016, Gill & Donsker 2017), although some do still classify it merely as a subspecies (*P. v. sharpei*) (Dickinson & Remsen 2013).

Morphologically, *sharpei* birds found in most of Portugal, Spain and southern France north to Hérault *département* (Figure 1) differ in facial colour (lores, supercilium to above eye, ocular and moustachial areas), being black in birds belonging to the European lineage (*P. viridis viridis* lineage) and grey in Iberian birds. Smaller differences are also present in other parts of the head plumage (mainly the crown) and in the pattern on the undertail coverts (Figure 2) (Svensson & Grant 2001, Winkler & Christie 2017). These two lineages have mostly similar calls but differ in the distinctive laughing call given during the breeding season, which is generally higher pitched, faster and differently structured in *sharpei* birds (Fauré 2013, Winkler & Christie 2017). These differences have thus been used in some taxonomies as the basis for the specific treatment of the Iberian Green Woodpecker following Tobias' criteria (Tobias *et al.* 2010, del Hoyo & Collar 2014).

Previous studies focusing on fragments of other mtDNA genes (CYTB and ND2), as well



Picus viridis viridis *Picus viridis sharpei* *Picus vaillantii*

Figure 2. Head pattern (females above, males below) of three different members of the European Green Woodpecker species complex based on Svensson & Grant (2001). Note the differences stated in the introduction, particularly black face of *Picus viridis viridis* birds vs grey face of those belonging to the *P. viridis sharpei* lineage; also note the greyer, more orange crown and duskier cheeks in latter.
Patrons de plomatge del cap (femelles dalt, mascles baix) de tres membres del complex del picot verd basats en Svensson & Grant (2001). Noteu les diferències descrites en l'apartat introductori de l'article, en particular la cara negra dels ocells pertanyents al llinatge Picus viridis viridis *que passa a ser grisa en aquells pertanyents al llinatge* P. viridis sharpei*; també destaca la corona de color més taronja i grisenca i les galtes més brutes del darrer tàxon.*

as several nuclear and mitochondrial introns (most notably the Z-linked BRM intron), have shown reciprocal monophyly between the Iberian Green Woodpecker and all other European Green Woodpecker populations, with the former lineage being determined as a sister group to the latter (Pons *et al.* 2011, Perktas *et al.* 2013). This, along with the high nuclear genetic differentiation found in more recent studies (Pons *et al.* 2019), suggests that the Iberian Green Woodpecker should be accorded specific status (Pons *et al.* 2011, 2019). It has been estimated that these lineages split about 0.7–1.2 million Ma, probably when they became isolated by geographical barriers that boosted allopatric divergence (Pons *et al.* 2011).

As previously commented above, the nominate subspecies and the Iberian Green Woodpecker are known to intergrade in a narrow area in southern France near the Mediterranean Sea, mainly in the north of the Aude and south of the Hérault *départements* (both SE France), where morphologically intermediate birds are found (Olioso & Pons 2011). Although these intermediate birds once put the specific status of the Iberian lineage in doubt, this hybridisation has been shown to occur only in a secondary contact zone across which there is little gene flow. The low level of genetic admixture of birds in this area suggests the existence of efficient isolating barriers between the two lineages, thereby supporting their splitting into different species (Pons *et al.* 2019).

The above-mentioned studies have also shown that genetic diversity within the European lineage of *P. viridis* is very low (0.1–0.2 times the diversity found between *sharpei* and *viridis*). By contrast, the Maghreb Green Woodpecker is more distantly related to other *P. viridis* populations (including *sharpei*), having diverged 1.6–2.2 Ma, and so is now regarded as a sister group to both the European and Iberian lineages (Pons *et al.* 2011).

Our aim was to obtain the DNA barcodes of the COI-5P region to fill the gap in the barcoding coverage of the distinctive Iberian Green Woodpecker (*P. viridis sharpei*) lineage. In addition, we compared these newly determined barcodes with those of the European populations of *Picus viridis* to assess whether or not the divergence rate fits the expected distance between species, given that their treatment as separate species is supported by other morphological and genetic evidence. This study also used the Generalized Mixed Yule Coalescent (GMYC) method for species delimitation to investigate species limits within this group.

Material and methods

a) Comparison of COI sequences

To compare the COI sequences of the two focal taxa in this study, DNA extraction and amplification of the COI-5P region was carried out for birds from the Iberian *sharpei* lineage; for individuals from the European lineage, twelve sequences (694-728 bp) were obtained from GenBank (*ncbi.nlm.nih.gov/genbank*), two of which belonged to *P. viridis karelini* (see Annex 1 for accession numbers).

Two DNA extractions per sample were taken from muscle tissue from the five *P. viridis sharpei* lineage birds obtained from locations 150–250 km apart (Figure 1; Annex 2) using a GeneJET® Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol for Mammalian Tissue Genomic DNA Purification.

The required DNA fragment was amplified using primers Bird F1 (TTCTCCAACCACAAAGACATTGGCAC) and Bird R1 (ACGTGGGAGATAATTCCAAATCC-TG). The following PCR conditions were used: 2 min at 95ºC; 45 cycles of 30 s at 95ºC, 30 s at 50ºC and 1 min at 72ºC; and 5 final minutes at 72ºC. PCR products were run in a 1.5% agarose gel. The purification of the PCR products (700 base pairs) of both replicas was performed by excising the band from the gels using QIAquick® Gel Purification Kit (QIAGEN) following the manufacturer's instructions. They were then quantified with a Nanodrop® spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and sequenced with Folmer primers (see above) by GATC Biotech (www.gatc-biotech.com), as per the manufacturer's specifications.

We obtained nine forward and nine reverse COI-5P traces from the five birds. The alignment gave five high-quality sequences, which were then uploaded into the BOLD system. In this portal, we checked whether or not they clustered around the same Barcode Index Number

(BIN) – an operational taxonomic unit used by BOLD systems that closely corresponds to species (Milton *et al.* 2013) – as the pre-existing *Picus viridis* sequences. Pairwise distances to other *Picus viridis* complex members, as well as to other picid species including the Grey-headed Woodpecker (*P. canus*), the most closely related species to the *Picus viridis* complex (Pons *et al.* 2011), were calculated by applying the Kimura-2-parameter (K2P) model. MEGA7 software (Kumar *et al.* 2016) for evolutionary analysis was used following the methods employed in previous studies (Hebert *et al.* 2004).

b) GMYC method for species delimitation

GMYC is a commonly used method of molecular species delimitation that employs an ultrametric tree to find the threshold dividing the between-species from the within-species branching process (Michonneau 2016, Ritchie *et al.* 2018). Although it has been shown to produce robust species hypotheses under a range of conditions, it has also been observed to over-split species, for example when speciation is rapid or when sampling does not adequately represent intraspecific diversity (Ritchie *et al.* 2016). Furthermore, although well-studied genes like COI and CYTB are commonly used in GMYC, their performance is questionable, as they can disagree on species delimitation (Ritchie *et al.* 2016). The use of this method in combination with other molecular species delimitation methods and additional morphological and ecological evidence is advisable, and has led to taxonomic revisions in some avian complexes (Ritchie *et al.* 2016, Marki *et al.* 2018).

Our analysis was carried out using both COI and CYTB. To ensure the greatest possible accuracy, we used the maximum number of samples we could find from a series of species. However, given that many were identical, which would bias the species delimitation method (Ritchie *et al.* 2016, Marki *et al.* 2018), we only retained one sequence/haplotype. This gave 12 COI sequences and 18 CYTB sequences consisting of all public BOLD sequences from the *Picus viridis* complex (which included our *sharpei* sequences but none from *P. vaillantii*), *P. canus* and *P. awokera* for COI, and all public GenBank sequences from the same taxa plus *P. vaillantii* for CYTB.

We largely followed Michonneau (2016), the only difference being our decision to set the chain length of the BEAST analysis to 10 million instead of 5 million steps. The trees were built under a Yule model and a constant clock, a decision made following BEAST guidelines that suggest that these assumptions are adequate for analysing a single locus involving only a few closely related species.

To improve the accuracy of the results, we repeated the analysis of each gene by incorporating calibration nodes using the divergence times between lineages of Green Woodpeckers proposed by previous studies (Pons *et al.* 2011). Accordingly, these divergence times were set to 0.7–1.2 Ma for the *viridis-sharpei* split, 1.6–2.2 Ma for *viridis-vaillantii* and 2–3.6 Ma for *viridis-canus*. The process was carried out following the recommendations of Drummond *et al.* (2013).

Results

In total, five sequences from five different individuals of Iberian Green Woodpecker were obtained and uploaded to the BOLD public data portal (Annex 2). The length of the sequence was optimal for analysis as it ranged between 664 bp and 715 bp and the divergence rate between them was null (0.00%). The slight difference in chain length only caused small alterations when compared to the other available *Picus viridis* sequences (despite the absence of divergence between these five sequences in their 664 bp common region) (Annex 1).

The genetic distances found between the *sharpei* and *viridis* sequences varied depending on the origin of the samples. The distances between Iberian birds and their nearest European counterparts (i.e. those from France and the Netherlands) was 16–17bp from the c. 700 bp of each sequence, that is, 2.04–2.44% according to the K2P substitution model (Table 1; Annex 1). More distant sequences such as from one of the Norwegian birds (GU571576.1) had up to 2.61% divergence (Table 1; Annex 1). The average difference between the *sharpei* birds and all the *viridis* birds was 2.22% (Table 1).

According to the data available in GenBank, differences between individuals from the nominate subspecies are relatively small, with all the birds, except for a Norwegian bird (GU571576.1), differing only by 0–0.32%

Table 1. Summary of K2P distances (minimum, mean and maximum) found between COI-5P sequences of the studied woodpecker taxa. The distance found between the European and Iberian lineages fits the expected distance between species (over 2% and over 10 times the difference found within the *P. viridis viridis* lineage). *Resum de les distàncies (mínimes, mitjanes i màximes) trobades entre les seqüències de les regions COI-5P de diferents tàxons de picot seguint el model K2P. La distància trobada entre els llinatges europeus i ibèrics encaixa amb la que esperaríem trobar entre diferents espècies, ja que es troba per sobre del 2% i és més de 10 vegades més gran que aquella trobada dins el llinatge europeu.*

	Min Distance (%)	Mean Distance (%)	Max Distance (%)
Within *P. viridis sharpei* lineage	0.00	0.00	0.00
Within *P. viridis viridis* lineage	0.00	0.18	0.64
***P. viridis viridis* lineage – *P. viridis sharpei* lineage**	2.04	2.22	2.61
P. viridis sharpei* lineage – *P. canus	4.20	4.64	4.60
P. viridis – P. canus	5.21	5.48	5.82

(0–2bp). Indeed, birds from the *karelini* subspecies differed by only 0.16% from the nearest birds of the nominate subspecies (Annex 3). The Norwegian bird (GU571576.1) had 0.32–0.64% divergence from the other birds of the nominate subspecies. Mean intraspecific variation between all the available sequences of *Picus viridis* (excluding those from *sharpei* birds) was 0.18% (Table 1). The comparison of the sequences of *viridis* and *sharpei* birds with those of other species showed that *sharpei* birds differed by 4.64% from the COI-5P region in *P. canus*, while *viridis* birds differed from *P. canus* by an average of 5.48%.

All new *sharpei* sequences were assigned a new BIN (BOLD: ADO4780) that differs from the one to which all the other *P. viridis* barcodes had previously been assigned (BOLD: AAD1222). Phylogenetic trees verify the fact that Iberian birds form a clade that is distinct from all other Eurasian Green Woodpeckers (Pons *et al.* 2011, Perktas *et al.* 2013).

All GMYC analysis agreed on the species delimitation: those using COI suggested the existence of four species (*Picus viridis, P. sharpei, P. canus* and *P. awokera*), while those using CYTB also suggested the specific status of *P. vaillantii* (not included in the COI analysis). In those analyses that did not incorporate calibration nodes, the tree using CYTB placed *P. vaillantii* as sister to a group comprising *P. viridis, P. sharpei* and *P. canus*, an arrangement that does not coincide with that proposed by Pons *et al.* (2011). This discrepancy was not found when using the calibration nodes described in *Materials and methods*, which placed *P. vaillantii* as a sister to the clade formed by *P. viridis* and *P. sharpei* (Figure 3).

Discussion

As previously shown, our results reveal 2.04–2.61% genetic divergence in the COI-5P region between the Iberian Green Woodpecker and its nearest European relatives. This difference in this gene region exceeds the 1.25% variation that has been identified as the maximum average intraspecific divergence of most North American birds, and also surpasses the 2% minimum variation between the COI-5P region in several sister species found in the same study (Hebert *et al.* 2004, Kerr *et al.* 2007). This divergence rate is similar to that found between the same regions of other closely related species of woodpeckers known to occasionally hybridise but which are habitually recognized as full species: Ladder-backed *Dryobates scalaris* and Nuttall's *D. nuttallii* Woodpeckers (1.17–1.36% between closest populations) and Great Spotted *D. major* and Syrian *D. syriacus* Woodpeckers (2.63% divergence, although only one *syriacus* sequence was available). The mean intraspecific variation between birds of Iberian and European lineages (2.22%) was more than 10 times the mean intraspecific variation within European individuals (0.18%), thereby exceeding the threshold that has been proposed in other studies for recognizing provisionally divergent taxa as good species (Hebert *et al.* 2004). Therefore,

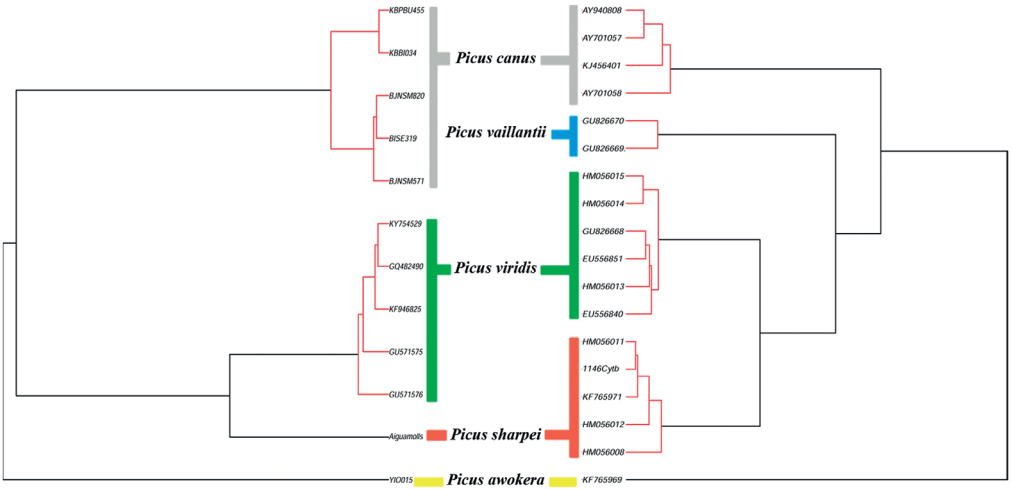


Figure 3. Phylogenetic trees resulting from the BEAST and posterior GMYC analyses for the COI-5P region (left) and CYTB (right) sequences with individual clusters highlighted in red. These specific trees were obtained using the calibration nodes described in the *Materials and methods* section of this article. Note that both trees delineate the Iberian lineage as a separate species (*Picus sharpei*). *P. vaillantii* only appears in the CYTB tree because there were no COI-5P samples available for this taxon.
Arbres filogenètics resultants de les anàlisis BEAST i GMYC de seqüències de la regió COI-5P (esquerra) i de CYTB (dreta), amb clústers individuals destacats en vermell. Aquests arbres han estat obtinguts utilitzant els nodes de calibratge descrits en l'apartat de Materials i mètodes *de l'article. Ambdós arbres delimiten el llinatge ibèric com una espècie diferent de la resta (*Picus sharpei*). Cal destacar que* P. vaillantii *apareix només en l'arbre del CYTB perquè no hi havia mostres de la regió COI-5P disponibles per aquest tàxon.*

the genetic distance found between the COI-5P regions of the Iberian and European lineages of Eurasian Green Woodpeckers fits the expected divergence between different species, which matches the assignment of the two lineages into different BINs.

Our GMYC analysis also provided insights into the relationship between Eurasian and Iberian Green Woodpeckers by confirming the consistent separation of these two taxa as different species. Even though these results support the specific treatment of the Iberian Green Woodpecker, the GMYC method has sometimes been shown to be unreliable when delimiting species, especially in cases of the incomplete sampling of large and variable populations, rapid speciation processes, or when some of this method's simplifying conventions such as the assumption of zero extinction or no hybrid introgression are violated (Ritchie *et al.* 2016). Such issues could lead to the oversplitting of the studied taxa. Nevertheless, the results of our study show that the GMYC analysis is an important indicator of the distinctiveness of the Iberian Green Woodpecker.

The newly found high divergence in the COI-5P region, together with the indications of the differentiation of the Iberian and Eurasian Green Woodpeckers according to both the BIN assignment and the results of the GMYC analysis, supports the specific status of the Iberian Green Woodpecker. However, as previously stated, both of the techniques used in this article for species delimitation have their exceptions and limitations and it has been argued that neither should be used alone as justification for splitting taxa into different species. Despite these limitations, our findings do fall in line with the most recent studies supporting the split of these two lineages as biological species (Pons *et al.* 2019). This treatment has already been adopted by some checklists (del Hoyo & Collar 2014, BirdLife International 2016, Gill & Donsker 2017), although others still consider the Iberian lineage as a subspecies (Dickinson & Remsen 2013). Taking into account our findings and the recent studies of the reproductive isolation of these two taxa, along with the consistent morphological and vocal differences discussed in the introduction, we support the universal

treatment of the Iberian Green Woodpecker as a species: *Picus sharpei*.

One of the main problems our research faced was the small number of available specimens. Indeed, sequences were only obtained from five individuals, all of which originated from a relatively small area (covering less than 10% of the Iberian Green Woodpecker's range). This small range of samples means that the amount of interspecific variation in the COI-5P region within the Iberian Green Woodpecker is still virtually unknown. Although the lack of morphological variation within this taxon suggests that the variation in the aforementioned region in birds from the Iberian Peninsula may in fact be small, further studies using more geographically distant samples are still needed to corroborate this hypothesis since great intraspecific variation could, for example, lead to changes in the results of the GMYC method.

As mentioned above, divergence in the COI sequences of birds from the European lineage were small, which fits perfectly with what is to be expected from conspecific individuals. Even birds from ssp. *karelini* differed only by 0.16% (1 bp) in the COI-5P region from those of the nominate subspecies, thereby falling within the range of variation found in the latter; this fact, along with previous evidence of genetic uniformity (Pons *et al.* 2011), could lead to the merging of ssp. *karelini* with the nominate subspecies if morphological differences are not consistent. However, even greater divergence is found in the Norwegian bird mentioned in the results section of the article (0.32–0.64% divergence in the studied region relative to other nominate birds). Although this may be attributable to a number of factors (including methodological reasons), the existence of a genetically differentiated population of birds assigned to the nominate subspecies in southern Scandinavia cannot be ruled out since slight divergence has been found in other mtDNA regions (Pons *et al.* 2011). Given that species delimitation based on the divergence between COI-5P barcodes is dependent on the intraspecific variation within species, high variation within the European lineage may affect our interpretation of the divergence found between the studied lineages. Therefore, a more complete sampling of the Eurasian Green Woodpecker, including Scandinavian birds and birds from ssp. *innominatus*, is needed in order to account for any COI divergence that may not have been considered in this study.

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Resum

Divergència entre les regions COI-5P dels llinatges europeu i ibèric del picot verd *Picus viridis*

Fa temps que es coneixen les significants diferències morfològiques del llinatge ibèric del picot verd *Picus viridis sharpei* respecte la subespècie nominal *Picus viridis viridis*. Diferents estudis basats en l'ADN mitocondrial de l'espècie han apuntat cap a la possibilitat d'elevar aquest llinatge al nivell d'espècie (*Picus sharpei*), un tractament ja adoptat per diferents taxonomies. Recentment també s'ha determintat que l'intercanvi genètic entre els dos llinatges en les zones on entren en contacte és limitat, fet que també dona suport a la separació dels dos llinatges en dues espècies. En aquest treball hem investigat si la divergència entre la regió COI-5P dels dos tàxons, un marcador d'ADN mitocondrial molt utilitzat en el diagnòstic d'espècies, encaixa amb la taxa de divergència esperada en espècies diferents. També hem utilitzat el mètode Generalized Mixed Yule-Coalescent (GMYC) per a la delimitació d'espècies per a determinar els límits específics dins d'aquest grup. Els resultat mostren una elevada divergència en aquesta regió (2,04% - 2,61%) entre els dos llinatges

i el mètode GMYC delimita el llinatge ibèric com una espècie diferent. Aquests resultats coincideixen amb aquells que esperaríem trobar en diferents espècies i, tenint en compte també els estudis previs sobre morfologia i aïllament reproductor entre els llinatges, donen suport a la separació del picot verd ibèric com a espècie.

Resumen

Divergencia entre las regiones COI-5P de los linajes europeo e ibérico del pito real *Picus viridis*

Hace tiempo que se conocen las significativas diferencias morfológicas del linaje ibérico del pito real *Picus viridis sharpei* respecto a la subespecie nominal *Picus viridis viridis*. Diferentes estudios basados en el ADN mitocondrial de la especie han apuntado hacia la posibilidad de elevar dicho linaje al nivel de especie (*Picus sharpei*), tratamiento ya adoptado por distintas taxonomías. Recientemente también se ha determinado que el intercambio genético entre ambos linajes en las zonas donde entran en contacto es limitado, hecho que también respalda su separación en especies distintas. En este artículo se ha investigado si la divergencia entre la región COI-5P de los dos taxones, un marcador de ADN mitocondrial muy utilizado en el diagnóstico de especies, encaja con la tasa de divergencia esperada entre diferentes especies. También hemos utilizado el método Generalized Mixed Yule-Coalescent (GMYC) para la delimitación de especies para determinar los límites específicos dentro de este complejo. Los resultados muestran una elevada divergencia en esta región (2,04%-2,61%) entre los dos linajes y el método GMYC delimita el linaje ibérico como una especie diferente. Estos resultados coinciden con aquellos que esperaríamos encontrar ente diferentes especies y, teniendo en cuenta también los estudios previos sobre la morfología y el aislamiento reproductor entre los linajes, apoyan la separación del pito real ibérico como especie.

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Annex 1. Divergence rates (%) between the COI-5P region of the five different individuals of Iberian Green Woodpecker (*Picus viridis sharpei* lineage) whose DNA was extracted, and the sequences of European Green Woodpecker (*P. viridis viridis* lineage) available at BOLD Systems. Note that, although they are identical, the sequences of the Iberian birds (ZSM-GI-0001 to ZSM-GI-0005) differ in various rates from the European sequences due to the differences in the length of the Iberian sequences. The sequences from the European lineage belong to two different subspecies, *P. viridis viridis* (PVV) and *P. viridis karelini* (PVK).
Taxa de divergència (%) trobada entre la regió COI-5P dels 5 individus de picot verd ibèric (llinatge Picus viridis sharpei*) dels quals es va extreure ADN i les seqüències de picot verd (llinatge* P. viridis viridis*) presents a BOLD Systems. Destacar que, tot i que siguin idèntiques, les cinc seqüències d'ocells ibèrics (ZSM-GI-0001 fins ZSM-GI-0005) divergeixen de les seqüències europees amb diferents taxes degut a les diferències de llargada entre les seqüències ibèriques. Les seqüències del llinatge europeu pertanyen a dues subespècies:* P. viridis viridis *(PVV) i* P. viridis karelini *(PVK).*

GenBank ID	Taxa	ZSM-GI-0001/2	ZSM-GI-0003	ZSM-GI-0004/5
GU571575	PVV	2.28	2.04	2.27
GU571576	PVV	2.61	2.22	2.61
GU566429	PVV	2.28	2.04	2.27
GQ482489	PVK	2.61	2.39	2.61
GQ482490	PVK	2.61	2.39	2.61
GQ482487	PVV	2.44	2.22	2.44
GQ482488	PVV	2.44	2.22	2.44
KF946824	PVV	2.44	2.22	2.44
KF946825	PVV	2.28	2.04	2.27
KF946826	PVV	2.44	2.22	2.44
KF946827	PVV	2.44	2.22	2.44
KF946828	PVV	2.28	2.04	2.27

Annex 2. Identification number and main characteristics of the Iberian Green Woodpecker individuals from which DNA was extracted.
Codis d'identificació i principals característiques dels individus de picot verd ibèric dels quals es va extreure ADN.

ID Number	Sex	Age	Locality	Date of collection	BOLD sample ID
T28610	Male	Juvenile	Puiggròs, Lleida, W Catalonia	15/07/2016	ZSM-GI-0001
AE.16.268	Male	Juvenile	Girona, NE Catalonia	22/06/2016	ZSM-GI-0002
MZB 2011-1146-T	Male	Juvenile	Gavà, Barcelona, E Catalonia	03/07/2011	ZSM-GI-0004
MZB 2010-1215-T	Male	Juvenile	Sant Pere Pescador, Girona, NE Catalonia	21/08/2010	ZSM-GI-0005
MZB 2010-0078-T	Female	Adult	Lladorre, Lleida, NW Catalonia	17/05/2009	ZSM-GI-0003

Annex 3. Estimates of the evolutionary divergence between the sequences (%) of different European and Iberian Green Woodpeckers and related species. The five first sequences (1-5) correspond to Iberian Green Woodpeckers (PVS) collected in Catalonia and were obtained through the process described in this paper, while most of the other sequences (6–16) correspond to two subspecies of European Green Woodpecker: *Picus viridis viridis* (PVV) and *P. viridis karelini* (PVK); the remaining two sequences correspond to two other species of the same genus: *P. canus* (PC) and *P. awokera* (PA). The origin and GenBank accession numbers of the sequences are specified in the table. All divergence rate estimates were obtained using the Kimura-2-parameter (K2P) model (Kimura 1980); the analysis employed 20 nucleotide sequences and all positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016). *Taxa de divergència (%) estimada entre els diferents individus de picot verd, picot verd ibèric i altres espècies relacionades. Les 5 primeres seqüències (1-5) pertanyen als individus de picot verd ibèric (PVS) recollits a Catalunya i van ser obtingudes seguint el procés especificat en aquest article, mentre que la majoria de la resta pertanyen a dues subespècies del picot verd:* Picus viridis viridis *(PVV) i* P. viridis karelini *(PVK); les* últimes *dues seqüències pertanyen a altres dues espècies del mateix gènere:* P. canus *(PC) i* P. awokera *(PA). A la taula s'especifica el lloc d'origen i el codi d'identificació de les seqüències a GenBank. Totes les taxes de divergència van ser obtingudes utilitzant el model K2P (Kimura 1980); l'anàlisi inclou 20 seqüències de nucleòtids i totes les posicions que contenien espais buits i falta de dades han estat eliminades. Aquestes anàlisis evolutives es van realitzar amb MEGA7 (Kumar* et al. *2016).*

	GenBank ID	Taxa	Location	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	ZSM-GI-0001	PVS	Lleida, ES																		
2	ZSM-GI-0002	PVS	Girona, ES	0.00																	
3	ZSM-GI-0003	PVS	Lleida, ES	0.00	0.00																
4	ZSM-GI-0004	PVS	Girona, ES	0.00	0.00	0.00															
5	ZSM-GI-0005	PVS	Barcelona, ES	0.00	0.00	0.00	0.00														
6	GU571575	PVV	Oslo, NO	2.06	2.06	2.06	2.06	2.06													
7	GU571576	PVV	Oslo, NO	2.23	2.23	2.23	2.23	2.23	0.17												
8	GU566429	PVV	FR	2.23	2.23	2.23	2.23	2.23	0.17	0.34											
9	GQ482489	PVK	Caucasus, RU	2.41	2.41	2.41	2.41	2.41	2.34	0.51	0.17										
10	GQ482490	PVK	Caucasus, RU	2.41	2.41	2.41	2.41	2.41	2.34	0.51	0.17	0.00									
11	GQ482488	PVV	Moscow, RU	2.23	2.23	2.23	2.23	2.23	0.17	0.34	0.00	0.17	0.17								
12	GQ482487	PVV	Moscow, RU	2.23	2.23	2.23	2.23	2.23	0.17	0.34	0.00	0.17	0.17	0.00							
13	KF946824	PVV	N Bravant, NL	2.23	2.23	2.23	2.23	2.23	0.17	0.34	0.00	0.17	0.17	0.00	0.00						
14	KF946825	PVV	Overjissel, NL	2.06	2.06	2.06	2.06	2.06	0.34	0.51	0.17	0.34	0.34	0.17	0.17	0.17					
15	KF946826	PVV	N Holland, NL	2.23	2.23	2.23	2.23	2.23	0.17	0.34	0.00	0.17	0.17	0.00	0.00	0.00	0.17				
16	KF946827	PVV	Limburg, NL	2.23	2.23	2.23	2.23	2.23	0.17	0.34	0.00	0.17	0.17	0.00	0.00	0.00	0.17	0.00			
17	KF946828	PVV	N Holland, NL	2.06	2.06	2.06	2.06	2.06	0.34	0.51	0.17	0.34	0.34	0.17	0.17	0.17	0.00	0.17	0.17		
18	AB843089	PC	Hokkaido, JP	4.37	4.37	4.37	4.37	4.37	5.11	5.30	5.30	5.48	5.48	5.30	5.30	5.30	5.11	5.30	5.30	5.11	
19	AB843698	PA	Honshu, JP	5.47	5.47	5.47	5.47	5.47	5.47	5.66	5.66	5.47	5.47	5.66	5.66	5.66	5.47	5.66	5.66	5.47	5.67