DNA analysis supports the presence of *Pontia edusa* (Fabricius, 1777), *Zizeeria karsandra* (Moore, 1865) and *Polyommatus celina* (Austaut, 1879) in Malta: A seasonal and multi-location investigation with additional notes on the central Mediterranean area

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SUMMARY

On the sole basis of morphometric analysis, it was routinely presumed that *Pontia daplidice* (Linnaeus, 1758), *Zizeeria knysna* (Trimen, 1862) and *Polyommatus icarus* (Rottemburg, 1775), occur in Malta. Recent DNA-based investigations (in-part combined with morphometric analysis) on islands and continental landmasses in the central Mediterranean shed light on the phylogeography of these and other, closely related, species. The present contribution focuses on seasonal and multi-location investigations within Malta of the genera *Pontia*, *Zizeeria* and *Polyommatus*. Voucher specimens from Sicily, Lampedusa and Spain are also analysed. A total of 43 specimens, in the following configuration are examined: *Pontia* ($n = 21$), *Polyommatus* ($n = 11$), *Zizeeria* ($n = 11$). DNA results obtained confirm the presence in Malta of *Pontia edusa* (Fabricius, 1777), *Zizeeria karsandra* (Moore, 1865) and *Polyommatus celina* (Austaut, 1879); conversely, *Z. knysna* and *P. icarus* are not reported from within the suite of specimens sequenced, while the presence of *P. daplidice* is confirmed from Lampedusa and, predictably, from the arid Monegros region in Spain.

INTRODUCTION

In view of their charismatic merit, butterflies provide exceptional prospect for environmental awareness, nature conservation and the promotion of citizen science. It is however essential that scientists, professional and amateur alike, work assuredly with species that have been accurately identified. Taxonomic research underpins basic biological sciences and applied disciplinary fields. Morphometric analysis was, for centuries, the conventional method utilised for taxonomic determination. However, a variety of methodologies involving DNA analysis have, in recent decades, been employed for taxonomic purposes and related reference systems, as well as for research in ecology, functional trait evolution and biodiversity conservation (Tautz et al. 2003; Kress et al. 2015). Moreover, such techniques have demonstrated that species hitherto thought to have occurred in a given location were, in reality, morphologically comparable or even visually identical, but nevertheless different species. An archetypal example that has pervaded the scientific literature for decades involves an error of misidentification, relating to *Danaus plexippus* (Linnaeus, 1758) and *D. genutia* (Cramer, [1779]) in India (Hamm, 2017)[1]. Situations such as these underscore the relevance of DNA characterisation and sequencing, which have since provided opportunities to examine the identity of species that cannot otherwise be determined by naked eye recognition and/or microscopy. More importantly, as articulated by Vodă et al. (2015a), molecular studies have been crucial in the recognition of a number of cryptic species, which, in view of their morphological similarities, had been overlooked. In particular, the authors draw attention to the relevance of cryptic taxa, whose distribution patterns are in many cases responsible for beta-diversity turnover in Europe, on both mainland and island environments. Consequently, the authors maintain that the frequency of replacement, or turnover, from location to location *"is recognised as a fundamental parameter in ecology and is widely used to detect biogeographic patterns"* (Vodă et al. 2015a, p. 405).

For centuries, it was thought that *Pontia daplidice* and *Polyommatus icarus*, and, more recently, *Zizeeria knysna*, occurred in the Maltese Islands as part of the indigenous suite of butterfly species (Valletta, 1966, 1972, 1974; Schembri, 1977; Sammut, 1982, 1984, 2000; Cassar, 2018). Relatively recent DNA-related investigations suggested, in effect, the presence of *Pontia edusa*, *Polyommatus celina* and *Zizeria karsandra* (Vodă et al. 2016). Further investigations carried out by the present authors on a series of specimens (taken during different seasons, principally during autumn and spring butterfly passage, and from a wide variety of geographical locations across Malta) affirmed an apparent absence of *P. daplidice, P. icarus* and *Z. knysna* (Cassar, 2018). The sequencing of material examined, in relation to this contribution, was initially carried out in collaboration with colleagues at the Institut de Biologia Evolutiva in Barcelona, and subsequently at the molecular genetics laboratories of the University of Malta. Based on our findings, it may be surmised that *P. edusa*, *P. celina* and *Z. karsandra* occur in the Maltese Islands.

MATERIAL EXAMINED

Specimens or selected body-parts thereof were used for this analysis, which comprised material from collections and as also fresh voucher specimens that were sampled specifically for the present study. Additionally, voucher specimens from overseas locations, notably from the Italian islands of Sicily and Lampedusa, and from mainland Spain, were also sequenced for

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[1] Classic cases in point, involving charismatic macro-mammals include the genomic analysis-driven split of the African elephant into two species (Rohland, N. et al. 2010) and, more recently, the split of the giraffe into four species (Fennessy et al. 2016).

purposes of comparability. Field sampling consisted of conventional stalking and netting techniques in suitable habitats, during known flight periods, for each of the species.

Research locations on the main island of Malta, as well as sample sites from within the central Mediterranean area (Sicily and Lampedusa), and from mainland Spain, together with their respective Global Positioning System (GPS) coordinates, are listed in Table 1.

The overall number of examined specimens, comprising the three genera sampled, was in total forty-three (TTL n = 43), represented as follows: *Pontia* (21); *Polyommatus* (11); *Zizeeria* (11). For a cartographical representation of the geographical spread of the voucher specimens sequenced, refer to Figure 1.

METHODS

The following methodology focuses mainly but not exclusively on the DNA analysis of 29 of the specimens - (the remaining 14 were analysed prior by the Institut de Biologia Evolutiva) - comprising the three genera under study. DNA was extracted from 29 ethanol-preserved specimens or parts thereof (excluding wings) using innuSPEED Tissue DNA Kit (Analytik Jena AG). The target 710 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 gene (COI) was amplified through polymerase chain reaction (PCR) using the standard barcoding primer pair LepF (5´-ATT CAA CA ATC ATA AAG ATA TTG G-3´) and LepR (5´-TAA ACT TCT GGA TGT CCA AAA AAT CA-3´) (Folmer et al. 1994).

Table 1. Geographical coordinates of sample sites.

|	
MALTA:	35°54'14" N 14°20'15" E – *Bahrija* valley slopes
	35°51'29" N 14°23'58" E – *Buskett*
	35°59'12" N 14°22'23" E – *Dahliet ix-Xilep, Ahrax*
	35°54'20" N 14°20'22" E – *Fomm ir-Rih*
	35°49'44" N 14°25'07" E – *Ghar Lapsi*
	35°49'53" N 14°27'34" E – *Maqluba, l/o Qrendi*
	35°56'38" N 14°25'12" E – *Salini*
	35°57'46" N 14°23'27" E – *Selmun*
	35°50'13" N 14°25'39" E – *Tal-Providenza, l/o Siggiewi*
	35°52'14" N 14°28'35" E – *Wied il-Kbir, Qormi*
	35°52'02" N 14°26'42" E – *Wied Qirda, l/o Haz-Zebbug*
	35°51'41" N 14°26'20" E – *Wied Qirda, l/o Siggiewi*
	35°49'40" N 14°27'48" E – *Zurrieq*
ITALY: LAMPEDUSA	35°30'47" N 12°37'15" E – *Cala Creta*
	35°29'49" N 12°37'34" E – *Cala Francese*
	35°30'25" N 12°36'44" E – north-eastern town periphery
	35°30'26" N 12°36'32" E – northern town periphery
	35°30'15" N 12°36'19" E – port area
	35°31'26" N 12°34'38" E – *Punta Cappellone*
ITALY: SICILY	37°06'31" N 15°03'19" E – *Borgo del Carato*
	37°58'47" N 13°55'32" E – *Collesano*
	37°06'58" N 14°56'22" E – *Valle dell'Anapo, Pantalica*
SPAIN:	41°48'30" N 0°29'02" W – *Monegros*
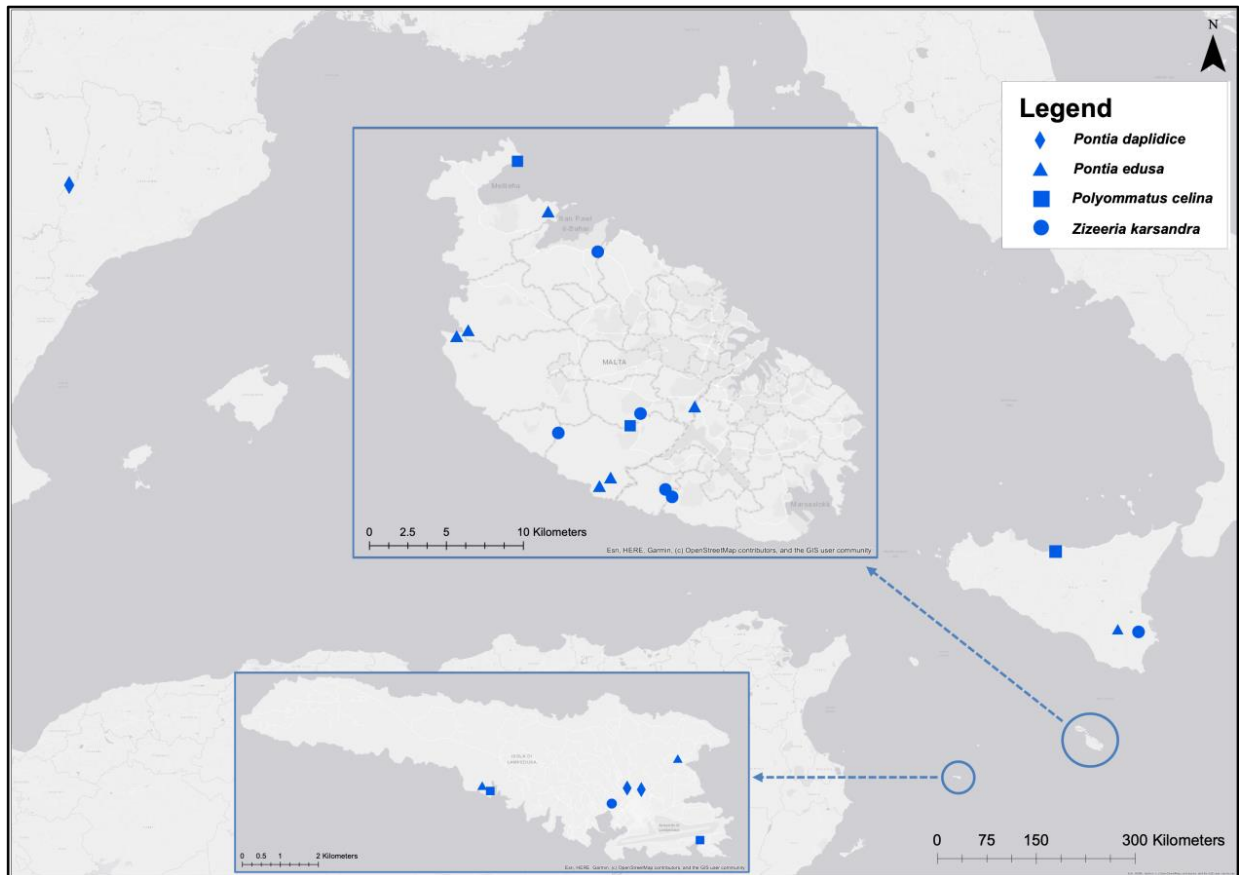
Figure 1. Research locations from where voucher specimens were collected for this study.

The following protocol was used for the 25 µL amplification reactions: 11.75 µL H₂O, 11.75 µL DreamTaq green PCR 2X Master Mix (Thermo Scientific), 0.5 µL of each 10 mM primer and 0.5 µL of DNA. The thermal profile used comprised initial denaturation for 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 50 s at 45 °C, 1 min at 72 °C, and a subsequent final extension at 72 °C for 5 min. Sanger sequencing was performed on the PCR products at GATC-biotech AG (Germany), using the same primers as in the PCR.

The sequence chromatograms were visually checked for any artefacts or heterogeneity. No double/superimposed peaks were detected. The COI sequence from each specimen was matched with barcode sequences on BOLD (Ratnasingham & Hebert, 2007) and subsequently uploaded on the same database. Ordinarily, a >2% sequence divergence at COI

in Lepidoptera would indicate taxonomic distinctiveness between taxa (Hebert et al. 2003b); a 2% pairwise distance threshold for species delimitation is also typically applied (Strutzenberger et al. 2011). For the barcode-based identity analysis, a threshold of 2% divergence was applied. Lepidoptera DNA barcoding for 1,030,371 specimens from 208 countries is currently available in the public domain through the BOLD database for sequence comparisons. Subsequently, MEGA-X (Kumar et al. 2018) was employed to appraise the level of taxonomic variation within the sequences analysed in this study. This included inferrment of evolutionary history, with the use of the Neighbour-Joining method (Saitou & Nei, 1987) to construct bootstrap consensus trees inferred from 1000 replicates (Felsenstein, 1985) and taken to represent the evolutionary histories of the specimens analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced

in less than 50% bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985) and evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Codon positions included were 1st+2nd+3rd+Noncoding and all positions containing gaps and missing data were eliminated.

RESULTS AND DISCUSSION

The suite of specimens of the genus *Pontia* that were analysed, whose geographical provenance comprised Malta and Sicily, were all confirmed to be *Pontia edusa*, as were the specimens collected from Lampedusa (taken at Punta Cappellone and Cala Creta during an apparent passage involving various species) in early April 2019. The two specimens collected from Lampedusa in early June 2021 and the specimen collected from the hyper-arid Monegros region of Spain were confirmed to be *Pontia daplidice* (see locations map represented in Figure 1).

The sequence of 17 of the *P. edusa* specimens analysed matched the dominant *P. edusa* COI sequence reported in the BOLD gene database (corresponding with sequences of the species extracted from specimens taken in Finland, Germany, Romania, Italy, and Sicily). The sequence of a *P. edusa* specimen from Borgo del Carato in Sicily differed from the rest by two synonymous nucleotides at COI loci 129 and 450; this haplotype was identical to the second dominant haplotype of *P. edusa* (reported in Italy and Sicily) recorded in the BOLD database, while a *P. edusa* specimen, taken at Cala Creta in Lampedusa in April 2019, differed from the rest at COI locus 567. In the case of *P. daplicide*, the identical sequences of the two Lampedusa specimens matched 100% with a COI sequence from a specimen taken in Cyprus, while the Monegros specimen, which differed from the other *P. daplidice* specimen sequences at loci 119, 137, 647 and 671,

matched 100% with a COI sequence of a specimen from Spain. In summary, intraspecific divergence values of sequenced *P. edusa* and *P. daplidice* specimens were, respectively, well within the 2% threshold. Furthermore, when compared with other species identified at other geographical locations, the level of intraspecific divergence was also very low.

The presence of *P. daplidice* in the Monegros is not an unexpected occurrence, since this region is well within the taxon's geographical area of distribution. However, it is of biogeographical significance to note that both *P. edusa* and *P. daplidice* have been recorded from Lampedusa. The island of Lampedusa lies some 130 km east of the Tunisian coast, its nearest landmass, a manageable distance (under suitable conditions) for various species of migratory butterflies. The presence of both taxa suggests that Lampedusa is either a zone of contact, where the two species may occur sympatrically, or that the two species are present on Lampedusa at different times of the year, potentially as visitors during seasonal episodes of multigenerational passage. Nonetheless, it can be ascertained that *P. daplidice* does breed on the island after a female specimen was taken (June 2021) while in the process of egg-laying. This specimen subsequently continued to deposit ova in captivity, the resulting offspring of which, were reared.

The COI sequences of the series analysed showed a manifestly evident interspecific differentiation; in fact, *P. daplidice* and *P. edusa* are known to demonstrate an unequivocal barcode gap (Wiemers & Fiedler, 2007; John et al. 2013). However, given the small sample size analysed from Lampedusa, further investigations are warranted in respect to these taxa, not only from the point of view of their respective occurrence period on the island and dispersal patterns across the central Mediterranean area, but also in terms of interspecific competition. Although the phylogenetic dynamics that occur in remote oceanic island chains (Waters, 2011; Waters et al. 2013) may seem incomparable with the

Lampedusa scenario, mainly because the largest island of the Pelagian group is not too distant from the African continental landmass for species such as *Pontia edusa* and *P. daplidice* to undertake the sea crossing, it would nonetheless be of interest to explore such relationships further. Such an investigation should be framed in a wider geographical context with a view to establish a broad understanding of post-glacial dispersal patterns and colonisation events within the central Mediterranean area.

All 11 sequenced specimens of the genus *Zizeeria*, which, apart from one individual specimen taken from the harbour region in Lampedusa, were collected in their entirety from different localities on the main island of Malta, were found to belong to the species *Z. karsandra*. Their extracted DNA sequences were all identical and aligned perfectly with the *Z. karsandra* sequence in the BOLD gene database (showing correspondence with sequences extracted from specimens collected in Pakistan and Thailand (Ashfaq et al. 2013; Chaianunporn & Chaianunporn, 2019). In spite of the geographical distance, it is interesting to note that no divergence in the DNA sequence in the database is evident. The occurrence of *Z. karsandra* in the Maltese Islands is of significant interest, given that the species is of north African affinity (in addition to its extensive eastern distribution). Its presence in the Maltese Islands, located north of the Pantelleria rift (comprising a graben complex which separates the Hybleo-Maltese platform from the Lampedusa plateau), attests to an intriguing yet important biogeographical connection for at least two reasons. First, the prevalent school-of-thought maintains that the last time the Maltese Islands were connected with the north African mainland was during the Messinian Salinity Event (Hsü et al. 1973; Hunt & Schembri, 1999; Cassar et al. 2008), and that subsequent species turnover occurred predominantly as a result of Pleistocene epoch lowstands when the Maltese archipelago was consequently connected with Sicily via an isthmus. In this context, the occurrence of *Z. karsandra* in the Maltese

Islands presents a glaring contrast biogeographically. Second, in a relatively recent review of Italian red-list butterflies (Bonelli et al. 2018), it was stated that the species' presence in Sicily was unlikely. Its occurrence in Lampedusa and the Maltese Islands, both emergent parts of the Pelagian Block, lying on the foreland margin of the African plate, but on opposite flanks of the Pantelleria Rift system that divides the central Mediterranean area, is thus intriguing and most certainly necessitates further investigation.

The confirmed presence of *Z. karsandra* on either side of the geographical N-S divide (created by the Pantelleria graben) bears broader ramifications on the contemporary understanding of the biogeography of central Mediterranean area. Accordingly, dispersal patterns and turnover events within this part of the Basin may not be as clear-cut as may ordinarily appear. The evidence brought to bear by the respective *Z. karsandra* records tends to cast an element of doubt on the approach of basing species' presence on islands merely on distance from surrounding landmasses, when potentially, other mechanisms may have been wholly or partially responsible for facilitating connectivity between isolated landmasses. One such case, for example, that may require reconsideration is the suggestion to treat the only three specimens of the genus *Hipparchia* taken in Malta as *H. blachieri* solely on the basis of Malta's geographical proximity to Sicily (Sammut, 2000; Tshikolovets, 2011; Cassar, 2018).

Sequenced specimens of the genus *Polyommatus*, which were collected from Malta, from different geographical locations in Sicily (comprising the north-facing slopes of the Madonie range, where *P. celina* and *P. icarus* may potentially occur sympatrically, and the Hyblean Plateau), and from the island of Lampedusa, were all found to be *P. Celina*. Although results for this taxon did not show a strong intra-specific variation in the DNA sequence, low variations (in the region of 1 to 5 nucleotides) were detected. In fact, most of the

specimens demonstrated a degree of variation in the COI gene (as indicated in Table 2), even among individuals collected from a common site. When matched with *P. celina* sequences published in the BOLD gene database, concurrence was evident, with the strongest match corresponding to sequences of specimens from Sicily (≅ MALTA: ♂ - *Wied Qirda, Siggiewi*; SICILY: 2♂♂ - *Collesano*; 2♂♂ - *Borgo del Carato*) and Iberia (≅ MALTA: ♂ - *Dahliet ix-Xilep*; SICILY: ♂ - *Borgo del Carato*; LAMPEDUSA: ♂ 2♀♀), while one individual male specimen taken from *Wied Qirda,* l/o *Siggiewi* matched a specimen sequence in the database from Malta.

Table 2. The seven frameworks of the COI gene fragment identified in *Polyommatus celina*.

Locus:	121	136	196	208	298	310	388	395	580
ITALY: Sicily, Collesano – 2♂♂	G	G	C	G	C	A	T	G	G
ITALY: Sicily, Borgo del Carato – 2♂♂ MALTA: Wied Qirda – ♂	G	G	C	A	C	A	T	A	A
ITALY: Sicily, Borgo del Carato – ♂	G	G	C	A	C	A	T	G	G
ITALY: Lampedusa, Punta Cappellone –♀	G	G	C	A	C	A	T	G	A
ITALY: Lampedusa, Punta Cappellone –♀ MALTA: Dahliet ix-Xilep, Ahrax – ♂	A	A	C	A	C	G	C	A	G
ITALY: Lampedusa, Cala Francese – ♂	G	A	T	A	T	G	C	A	G
MALTA: Wied Qirda – ♂	G	G	C	A	C	A	T	A	G

Phylogenetic trees for the species investigated are represented in Figure 2 (A-D). Figure 2 C corroborates the distribution limits of *Polyommatus celina* and *P. icarus* suggested by Dincă et al. (2011) and Tshikolovets (2011). The presence of *P. celina* in the Maltese Islands, among other islands within the central Mediterranean area, attests to multiple colonisation events originating from Sicily and the Maghreb, specifically dispersal across the Siculo-Tunisian Sill and emergent parts of the Pelagian Block. The sea-crossing across the former area is effectively the shortest geographical distance between Cap Bon in Tunisia and the south-western coastline of Sicily, while the latter affords a stepping-stone function, resulting from the role played by the Pelagic and Maltese islands in providing connectivity and thus minimising the effect of isolation by distance. Overall outcomes obtained for all four taxa examined are summarised in Table 3, together with respective details of the location and time of year of collection.
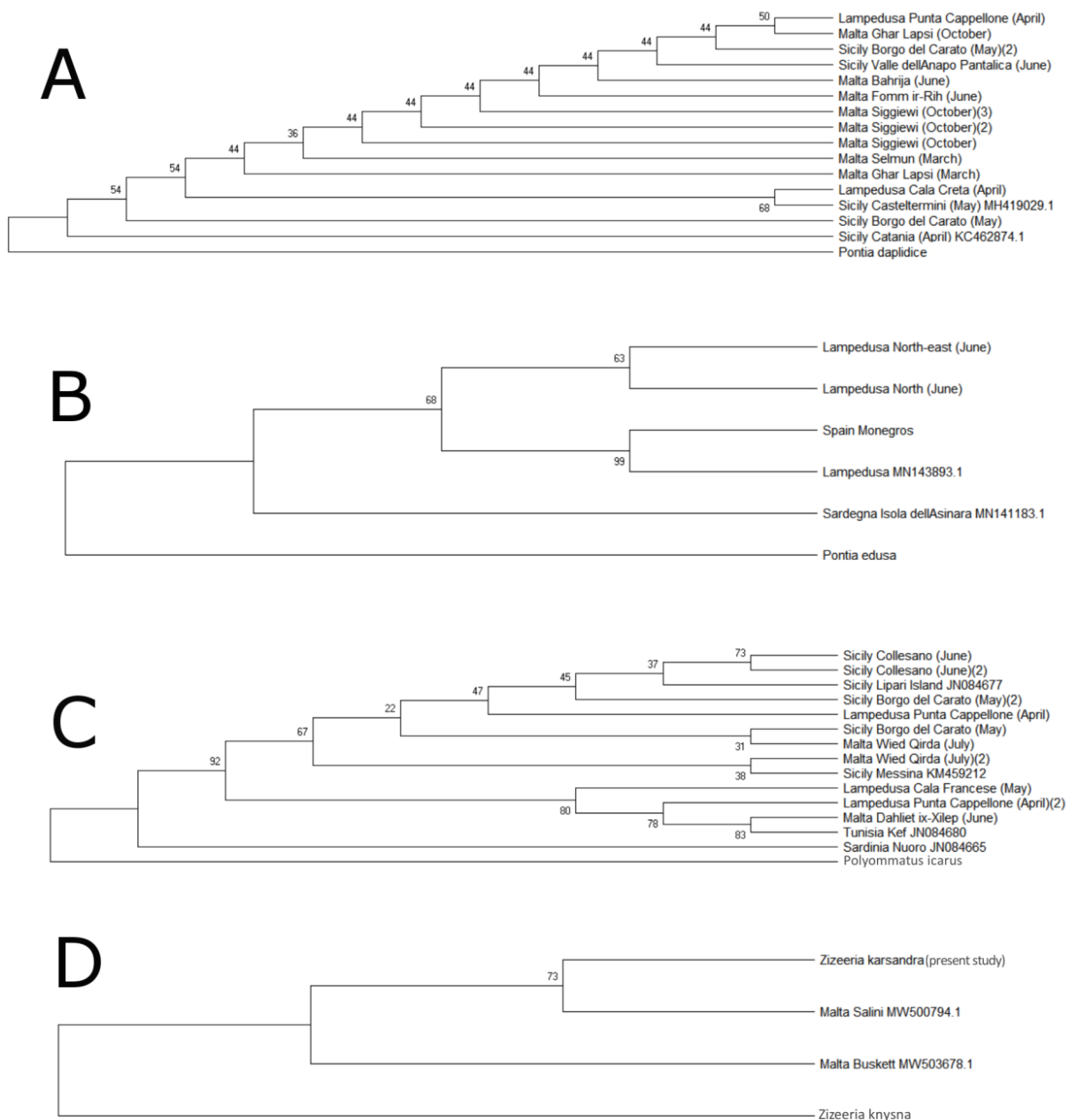
Figure 2 (A-D). Phylogenetic trees inferred using the Neighbour-Joining method (Saitou & Nei, 1987) for the specimens belonging to the four species investigated in the present study: (A) *Pontia edusa*: involving 13 nucleotide sequences from this present study, supplemented by two additional sequences from specimens originating from Sicily (Benson et al. 2013), with a total of 561 positions in the final dataset; (B) *Pontia daplidice*: involving 3 nucleotide sequences from this present study, supplemented by two additional sequences from specimens originating from Lampedusa and Sardegna (Benson et al. 2013), with a total of 596 positions in the final dataset; (C) *Polyommatus celina*: involving 10 nucleotide sequences from this present study, supplemented by four additional sequences from specimens originating from Lipari, Sicily, Tunisia and Sardegna (Benson et al. 2013), with a total of 536 positions in the final dataset; and (D) *Zizeeria karsandra*: involving 1 nucleotide sequence from this present study, supplemented by two additional sequences from specimens originating from Malta (Benson et al. 2013), with a total of 619 positions in the final dataset.

Table 3. Localities, month recorded and resulting species (where determined, sex is indicated)

Field collection data (location/month)	Species confirmed
Genus: *Pontia*	
MALTA: Bahrija – June	*P. edusa* ♂
MALTA: Fomm ir-Rih – June	*P. edusa* ♀
MALTA: Ghar Lapsi – March	*P. edusa* ♂
MALTA: Ghar Lapsi – October	*P. edusa* 5 ex.
MALTA: Wied il-Kbir (l/o Qormi) – October	*P. edusa*
MALTA: Selmun – March	*P. edusa* ♂
MALTA: Siggiewi – October	*P. edusa* ♂2♀♀
ITALY: Lampedusa, Cala Creta – April	*P. edusa* ♂
ITALY: Lampedusa, Punta Cappellone – April	*P. edusa* ♂
ITALY: Lampedusa, north-eastern town periphery – June	*P. daplidice* ♂
ITALY: Lampedusa, northern town periphery – June	*P. daplidice* ♀
ITALY: Sicily, Borgo del Carato – May	*P. edusa* 2♂♂
ITALY: Sicily, Valle dell'Anapo, Pantalica – June	*P. edusa* ♀
SPAIN: Monegros – September	*P. daplidice*
Genus: *Polyommatus*	
MALTA: Dahliet ix-Xilep, Ahrax – June	*P. celina* ♂
MALTA: Wied Qirda (l/o Siggiewi) – July	*P. celina* 2♂♂
ITALY: Lampedusa, Punta Cappellone – April	*P. celina* 2♀♀
ITALY: Lampedusa, Cala Francese – May	*P. celina* ♂
ITALY: Sicily, Borgo del Carato – May	*P. celina* 3♂♂
ITALY: Sicily, Collesano – June	*P. celina* 2♂♂
Genus: *Zizeeria*	
MALTA: Buskett – November	*Z. karsandra*
MALTA: Maqluba (l/o Qrendi) – September & October	*Z. karsandra* 3♂♂3♀♀
MALTA: Salini – October	*Z. karsandra*
MALTA: Wied Qirda (l/o Haz-Zebbug) – November	*Z. karsandra*
MALTA: Zurrieq – September	*Z. karsandra*
ITALY: Lampedusa, port area – June	*Z. karsandra*

FAMILY PIERIDAE

SUBFAMILY PIERINAE

Pontia edusa (Fabricius, 1777)

The combined range of *Pontia edusa* and *P. daplidice* extends from the Canary Islands, in the Atlantic, to the Mediterranean, across the Maghreb and the Middle East (*s.l.*)[2], as well as throughout significant parts of temperate and subtropical Asia. The species also occur across continental Europe, northward up to southern Scandinavia. Within the Mediterranean, the distribution of the two species can be broadly split between the western sub-basin and the Basin's central and eastern segments. *P. daplidice* occurs in North Africa, the Iberian Peninsula to the Pyrenees (eastern France) and NW Italy, although John et al. 2013 provide incontrovertible evidence, through molecular analysis, of its presence in Cyprus, Israel and Lebanon, suggesting an appreciably more widespread distribution. *P. edusa* is known to occur within the central and eastern parts of the Basin and Black Sea region. A measure of overlap is known to occur in parts of the Pyrenees, the NW mountainous regions of Italy and on the Liguria-Tuscany boundary, where introgressive hybridization has been reported (Porter, et al. 1997). As a result of clinal

[2] Egypt (inclusive of the Sinai), the Levant, Anatolia and Arabian Peninsula.

variation in allozyme markers, these authors had recommended the rank of subspecies for *P. edusa*, a deliberation also considered by others (Kurze et al. 2006), wherein the taxa are treated as a 'complex' (Kudrna et al. 2011).

It is clear that the two taxa cannot be distinguished from one another via straightforward, naked-eye examination of external characters or of male genitalia but can only be separated on the basis of biochemical and molecular analysis. In view of evident genomic divergence, we take the view of separate taxa, possibly occurring sympatrically in some regions (as is the case, potentially, in Lampedusa), especially but not exclusively during 'migration' episodes.

Within the Maltese Islands, *P. edusa* is a relatively common migrant species whose numbers are considerably augmented during spring and especially autumn passages. Although known to breed regularly, it is assumed that the local population is largely reinforced by such seasonal multigenerational influxes.

Up until relatively recently, it was presumed that *Pontia daplidice* occurred locally. In view of Malta's proximity to range boundaries of both taxa, it is not entirely discounted that *P. daplidice* may sporadically reach the Maltese Islands during passage events, as in the case of Lampedusa. This would effectively render the Maltese Islands a potential zone of contact for these two morphologically indistinguishable taxa.

FAMILY LYCAENIDAE

SUBFAMILY POLYOMMATINAE

Zizeeria karsandra (Moore, 1865)

The species has a fairly wide distribution, ranging from eastern Algeria to the Middle East and Turkey, extending eastward to tropical Asia and parts of northern and eastern Australia. Within the Mediterranean, in addition to its unbroken distribution from north-eastern Algeria to southern Turkey, it also occurs on parts of Sicily, on Malta, Crete and Cyprus, among other islands. Its close relative, *Z. knysna*, occurs on all of the Canary Islands, the southern segment of the Iberian Peninsula, Morocco and north-eastern Algeria. It also occurs in France, in an area northwest of the Pyrenees (Leraut, 2016).

Z. karsandra was recorded for the first time in the Maltese Islands in 1966, initially identified as *Z. knysna*, as were all local records until recently (Catania, 1992; Sammut, 2000; Cassar 2018). Subsequently, DNA analysis determined the presence of *Z. karsandra* (Vodă et al. 2016; Cassar, 2018). Over the years, it has been observed in a number of localities, where it appears to have established small, localised populations (Sammut, 2000; Cassar, 2018).

Polyommatus celina (Austaut, 1879)

The distribution of *P. celina* comprises the northern portion of the Maghreb as well as areas in central and southern Spain, the Balearic Islands, some of the Canary Islands, Sardinia, Sicily and some of the nearby islands (including Malta), and the Benghazi-Jebel Akhdar region of Libya. Its close relative, *P. icarus*, has a much wider distribution, comprising much of Eurasia, including the Levant, extending to the Far East. Recently, the species was discovered in eastern Canada, though it is thought to have been introduced. Various authors acknowledge extensive variation and the need for more taxonomical work (Tshikolovets, 2011; Leraut, 2016).

Within the Mediterranean Basin, it appears that the main barrier which separates these seemingly non-migratory taxa is the sea itself, even if, in some places, by exceedingly narrow marine causeways, as in the case of Corsica-Sardinia, Morocco-Iberia and, mainland Italy-Sicily. Generally, *P. icarus* is known to occupy the northern and eastern shores of the Basin, including the island of Crete, while *P. celina* occurs on the southern shores and islands within the western sub-Basin and central Mediterranean area. There is evidence of

established zones of contact in Spain (Tshikolovets, 2011; Leraut, 2016), where the two species, described as having "*deep genetic divergence*", appear to occur sympatrically (Dincă, et al. 2011).

In the Maltese islands, *P. celina* (which, until recently, was thought to be *P. icarus*) was considered a somewhat common species. However, it has declined markedly in recent years; whereas in the past it was possible to encounter scores of this butterfly within suitable habitats, individuals are only seen sparsely nowadays.

CONCLUSIONS

The Maltese Islands comprise a small low-lying group of Tertiary-age limestone isles that form part of the emergent elements of a complex foreland structure within the central Mediterranean, already referred to as the Pelagian Block. The Maltese Islands support one of the more impoverished butterfly faunas in the Mediterranean. This is due to various reasons, including the islands' relatively semi-arid climate and dearth of freshwater bodies, a restricted land-area and lack of habitat gradient (with maximum altitude of 253 m amsl), and a fairly uniform geology that produces a shallow and fundamentally calcareous soil profile. Furthermore, available habitat is negatively affected by substantial fragmentation resulting from agricultural practices, as well as the boundless spread of urbanisation and infrastructure. Notwithstanding, the Maltese island cluster lies on a geographical crossroads between southern Europe and North Africa, and, simultaneously, at the centre of an east-west Mediterranean axis.

Location and proximity are, in consequence, crucial factors affecting the Islands' present-day butterfly composition. Had it not been for recurring visitor influxes by species such as *Pieris rapae* (Linnaeus, 1758), *P. brassicae* (Linnaeus, 1758), *P. edusa, Colias croceus* (Geoffroy in Fourcroy, 1785), *Lampides boeticus* (Linnaeus, 1767), *Vanessa cardui* (Linnaeus, 1758), and *V. atalanta* (Linnaeus, 1758), which tend to boost population numbers from time to time, the butterfly fauna of the Maltese Islands would be far more impoverished indeed.

The islands' geographical location in the central Mediterranean area is thus of particular relevance to north-south multigenerational passage, while also pertinently positioned between the Mediterranean's eastern and western sub-basins. Mountainous terrain and open sea can be determinant extrinsic barriers to movement of species and where sub-populations are consequently separated, it follows that vicariant processes may potentially arise. This appears to be the case with the quasi-sedentary species *Polyommatus celina* and *P. icarus*, and, to some degree with *Zizeeria karsandra* and *Z. knysna*, whose respective distribution occurs within relatively separate geographical ranges, albeit with marginal exception (Tshikolovets, 2011; Leraut, 2016).

In view of the foregoing, as also due to the fact that DNA analysis often involves relatively limited sample numbers, further research may be warranted, particularly because of the proximity of the Maltese Island cluster to larger continental landmasses and other islands, with the latter potentially serving a stepping-stone function for potential connectivity.

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