*SHILAP Revista de lepidopterología* 53 (211) septiembre 2025: 429-437 eISSN: 2340-4078 ISSN: 0300-5267
https://doi.org/10.57065/shilap.1074 https://shilap.org
https://zoobank.org/5C3BC408-C8A0-4408-9CC0-9FE532A7CB21

# Expecting the unexpected: Random tissue barcoding reveals the presence of Pieridae in the diet of ground-dwelling Tenebrionidae (Insecta: Lepidoptera, Coleoptera)

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**Abstract**

The genus of Tenebrionidae (Coleoptera) *Oxycara* Solier, 1835 includes 16 species endemic to the Cabo Verde Archipelago. In this study we analysed part of the diet of *O. richardi* Alluaud, 1936, endemic to the island of Sal, through the non-targeted amplification of a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) from a specimen of this species. The results revealed that these detritivorous Coleoptera opportunistically feed on *Pontia glauconome* (Klug, 1829), a species of Pieridae also present in the archipelago. This finding provides new data on the trophic interactions and feeding behaviour of *O. richardi*.
**Keywords:** Insecta, Lepidoptera, Coleoptera, COI, Tenbrionidae, *Oxycara*, Pieridae, *Pontia*, Cabo Verde.

**Esperando lo inesperado: *barcoding* aleatorio de los tejidos revela la presencia de Pieridae en la dieta de Tenebrionidae terrestres (Insecta: Lepidoptera, Coleoptera)**

**Resumen**

El género de Tenebrionidae (Coleoptera) *Oxycara* Solier, 1835 incluye 16 especies endémicas del archipiélago de Cabo Verde. En este estudio analizamos parte de la dieta de *O. richardi* Alluaud, 1936, endémica de la isla de Sal, mediante la amplificación no dirigida de un fragmento del gen mitocondrial citocromo *c* oxidasa subunidad I (COI) de un ejemplar de esta especie. Los resultados revelaron que estos Coleoptera detritívoros se alimentan oportunistamente de *Pontia glauconome* (Klug, 1829), una especie de Pieridae también presente en el archipiélago. Este hallazgo aporta nuevos datos sobre las interacciones tróficas y el comportamiento alimentario de *O. richardi*.
**Palabras clave:** Insecta, Lepidoptera, Coleoptera, COI, Tenebrionidae, *Oxycara*, Pieridae, *Pontia*, Cabo Verde.

**Esperando o inesperado: *barcoding* aleatório de tecidos revela a presença de Pieridae na dieta de Tenebrionidae terrestres (Insecta: Lepidoptera, Coleoptera)**

**Resumo**

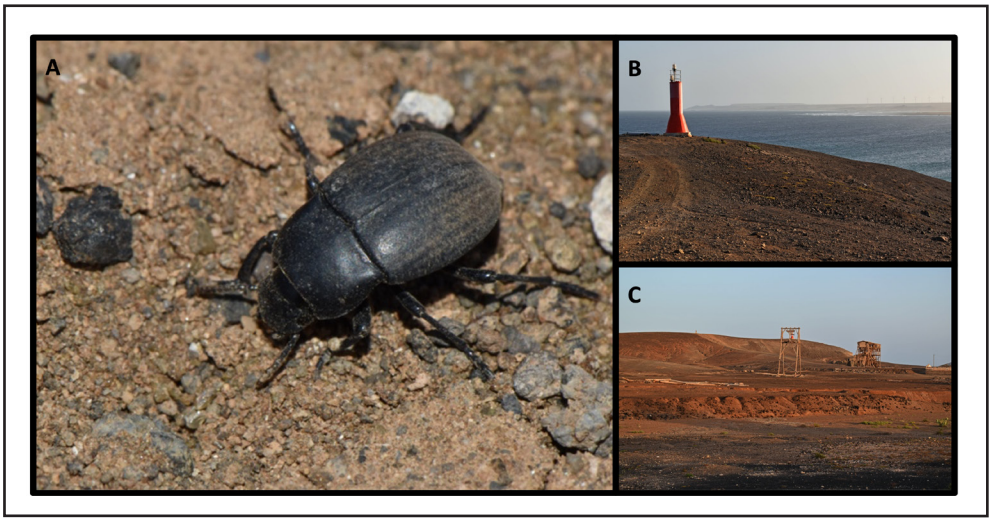
O género de Tenebrionidae (Coleoptera) *Oxycara* Solier, 1835 inclui 16 espécies endémicas do Arquipélago de Cabo Verde. Neste estudo analisámos parte da dieta de *O. richardi* Alluaud, 1936, endémica da ilha do Sal, através da amplificação não dirigida de um fragmento do gene mitocondrial citocromo *c* oxidase subunidade I (COI) de um exemplar desta espécie. Os resultados revelaram que estes Coleoptera detritívoros

se alimentam oportunisticamente de *Pontia glauconome* (Klug, 1829), uma espécie de Pieridae também presente no arquipélago. Esta descoberta fornece novos dados sobre as interações tróficas e o comportamento alimentar de *O. richardi*.
**Palavras-chave:** Insecta, Lepidoptera, Coleoptera, COI, Tenebrionidae, *Oxycara*, Pieridae, *Pontia*, Cabo Verde.

## Introduction

DNA barcoding is a versatile, useful, and important technique for monitoring and cataloguing biodiversity (Francis et al. 2010; Miller et al. 2016; Dincă et al. 2021), especially in hotspot areas where many endemic species are usually found, and cryptic species can hence be unveiled (Vasconcelos et al. 2016). This technique is thus crucial in under-studied sites, such as most African countries (Vasconcelos et al. 2016; Pereira et al. 2019; Gil et al. 2020; Pinho et al. 2024), and groups, such as invertebrates, which not only constitute the greatest diversity on earth, but also form the foundation of ecological chains (Shashank et al. 2022). DNA barcoding hinges on the comparison of individual DNA sequences with a reference library of known sequences (or DNA barcodes) linked to identified taxa, enabling precise identification of organisms (Hebert et al. 2003a, b; Ferri et al. 2009). This molecular method is especially useful when traditional species identification techniques are not applicable, such us with unknown or cryptic species (Hebert et al. 2004; Lara et al. 2010; Vasconcelos et al. 2016), developmental stages that are challenging to identify morphologically (Webb et al. 2006; Yeo et al. 2018; Chu et al. 2019) or parts or excretions (such as faeces) of organisms (Speller et al. 2016; Bennett et al. 2017; Dalén et al. 2017). The gene regions for effective barcoding depends on the group of organisms being worked with, as a good reference library is required, but certainly the mitochondrial cytochrome *c* oxidase subunit I (COI) is the most widely used, especially for animals and protists (Hebert et al. 2003a, b).

**Figure 1.** Study species: *Oxycara richardi* Alluaud, 1936 from Monte Grande (Sal, Cabo Verde) (photographed by PJ-A) **(A)**. Lighthouse **(B)** and Salterns of Pedra de Lume **(C)** (Sal, Cabo Verde), where specimens of *Oxycara richardi* Alluaud, 1936 were captured (photographed by MG-P).




Amplification of non-target organism is one of the biggest obstacles to DNA barcoding (Vargas et al. 2012), a problem that increases when universal COI primers are used (Mioduchowska et al. 2018; Leese et al. 2021). In many cases, barcoding of bacteria, parasites, or other organisms associated with the target species, such as their prey, are obtained (Smith et al. 2012; Mioduchowska et al. 2018; Pilgrim et al. 2021). In other cases, this obstacle can turn into an advantage, unveiling useful information about the species ecology (Pilgrim et al. 2021).

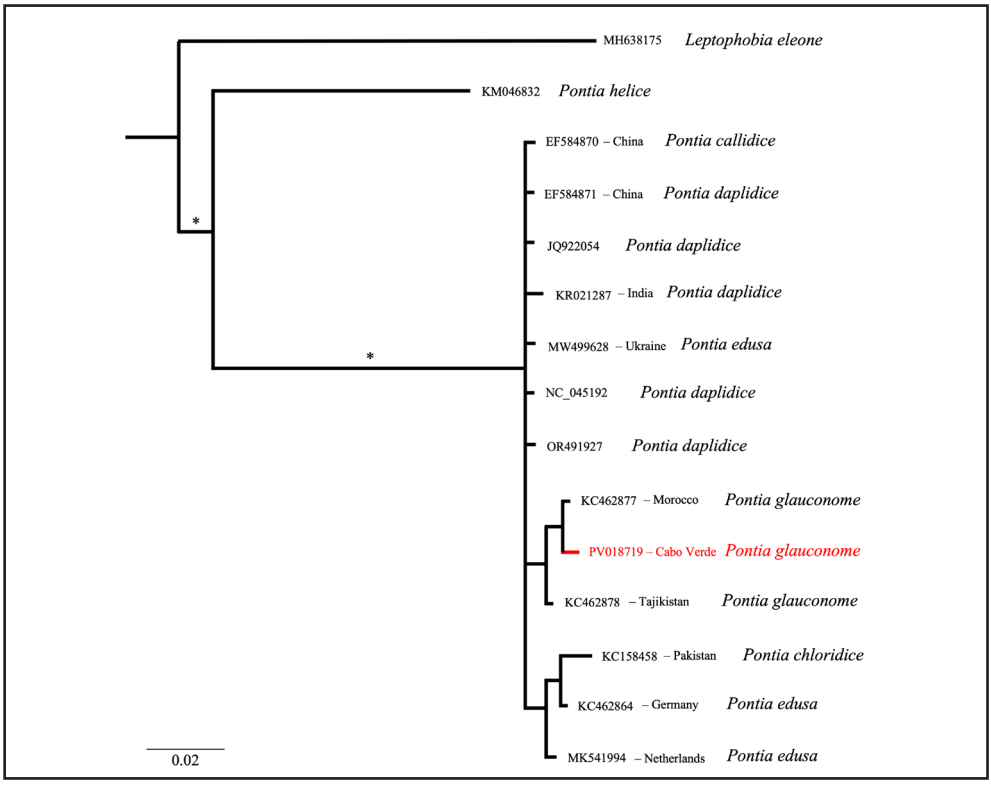
Thus, the goal of this article was to identify part of the diet of a darkling beetle *Oxycara richardi* Alluaud, 1936 (Figure 1A), endemic to the African insular country Cabo Verde that we failed to amplify, by amplifying some of what was probably its digestive content instead.

## Materials and Methods

Specimens of *Oxycara richardi* (Figure 1A), an endemic Tenebrionidae species of the island of Sal, Cabo Verde Island, were captured in January 2023 to study the distribution of the genetic diversity of this genus in this African country. The specimens were found in Pedra Lume, in the east part of the island, under stones near a lighthouse (Figure 1B), and next to the salterns (Figure 1C). Specimens were preserved in ethanol 96% and stored at -20ºC at the entomological collection of the Museo Nacional de Ciencias Naturales (MNCN-CSIC), Madrid, Spain.

DNA material was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and following the protocol described by the manufacturer. For DNA extraction from beetles, the coxa muscle or one or two legs of specimens is usually used (Sánchez-Vialas et al. 2020; Mas-Peinado et al. 2022; Jurado-Angulo et al. 2023). However, in the case of small taxa, usually the whole body is used (Sanz-LaParra et al. 2023). Thus, the whole specimen was used for DNA extraction, with a small perforation on the ventral part of the thorax. After incubation of the sample in Buffer ATL and proteinase K as indicated in the protocol, the specimen was recovered, washed in Milli-Q water and preserved again in ethanol 96% for morphological studies. This drilling technique is commonly used on small invertebrates (Sanz-LaParra et al. 2023).

**Figure 2.** Bayesian phylogenetic tree based on COI partial sequences. The sequences are identified by their GenBank codes, followed by their geographical origin when available, and their published taxonomic identification. The red colour indicates the sequence obtained in this study. Posterior probability (PP) values higher than 95% for the Bayesian analysis are represented by an asterisk (*) and are shown above nodes.



Polymerase chain reaction (PCR) was used to amplify a fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI), using the universal set of primers LCO1490 and HCO2198 (Folmer et al. 1994). PCR was performed in 25 µL, including 24 µL of the PCR mix (17.8 µL of H2O; 2.5 µL of a reaction buffer with MgCl2 (NZYtech); 1.5 µL of MgCl2; 1 µL of dNTPs; 0.5 µL of each primer and 0.2 µL of Taq polymerase (5U/ µL, NZYtech)) and 1 µL of specimen DNA. Amplification was conducted under the following PCR conditions: initial denaturation step at 95ºC (5 min), followed by 40 cycles of denaturation at 94ºC (45 seg), annealing at 42ºC (45 seg) and extension at 72ºC (1 min), and a final elongation step at 72ºC (10 min). Amplified product was visualized on a 1% agarose gel, and it was purified and sequenced by Sanger at Macrogen Inc. (Macrogen Europe, Madrid, Spain). Chromatograms of forward and reverse sequence were visualized, checked, and concatenated using Geneious Prime 21.1.1 (https://www.geneious.com). Consensus sequence was identified at the specific level using BLAST (Camacho et al. 2023) as belonging to the Pieridae *Pontia glauconome* (Klug, 1829). Since this result does not correspond to the expected one, which would be that the sequence was identified as *Oxycara*, and since the laboratory work was carried out with the pertinent care and no laboratory user works with Lepidoptera, we assume that it is not a contamination, but we amplified part of the diet of the studied specimen.

To identify the possible geographical origin of the butterfly consumed by *Oxycara*, a phylogenetic tree was reconstruct using this sequence, 13 GenBank sequences of *Pontia* Fabricius, 1807, and one additional sequence of *Leptophobia philoma* (Hewitson, 1870) used as closely related outgroup (Figure 2) (Ashfaq et al. 2013; Wahlberg et al. 2014; Dufour et al. 2018; Nie et al. 2018; Dincă et al. 2021).

Bayesian inference (BI) was conducted in MrBayes 3.2.6 (Ronquist et al. 2012). The command lset nst = mixed was used to determine the best substitution model. The BI consisted of two simultaneous runs of 20 million generations, sampling trees every 100 generations, and discarding the first 20% generations as burnin. Visualization and editing of the resulting phylogenetic tree were carried out in FigTree v.1.4.4 (Rambaut et al. 2018).

## Results

BLAST identified the amplified sequence as *Pontia glauconome* (GenBank accession number PV018719), with 99.85% and 99.70% ID (GenBank accession numbers KC462877 and KC46878, respectively). Based on the phylogenetic tree (Figure 2), the specimen from Cabo Verde was more closely related to specimens from this species from Morocco than from those available from Tajikistan. The three sequences of the species were more related to each other than to those of other species of *Pontia* Fabricius, 1807. However, not all the sequences included depicted monophyletic taxa. The mitochondrial phylogenetic tree reflects the poor diversification of the COI in this species group (Figure 2).

## Discussion

The DNA barcoding of a *O. richardi* specimen from Sal, Cabo Verde, did not provide the expected results. Instead, *Pontia glauconome* was amplified, suggesting that this species of Lepidoptera is part of the diet of the species. DNA amplification of food items was possible because, as the abdomen of the darkling beetle was perforated, DNA from its digestive contents was also extracted. Instead of considering this as a setback in the aimed phylogenetic study, this result was investigated as it highlights how DNA barcoding may have significant applications in ecological studies, particularly in diet analyses when morphological identification is compromised (Pompanon et al. 2012; Pinho et al. 2022, 2023).

Different parts of organisms can be used for diet analyses using DNA (meta) barcoding, such as stomachs (Harms-Tuohy et al. 2016; Pinho et al. 2024), faeces (Santos et al. 2022), saliva (Nichols et al. 2015), or the entire bodies (Macías-Hernández et al. 2018). The latter is used with small animals (Lynggaard et al. 2021). The total medium length of *Oxycara richardi* is approximately 1 cm, including head, thorax, and abdomen (Figure 1A). In addition, for the correct preservation of tenebrionid DNA, the specimens must be injected with alcohol, resulting in a perforation in the area where the needle is introduced, usually the anterior portion of the abdomen (Mas-Peinado et al. 2015, 2021, 2022).

*Pontia glauconome* is a Pieridae inhabiting xerophytic areas in North Africa, Arabian and Middle Eastern deserts, also recorded in Cabo Verde, specifically on Fogo, Maio, Boavista and Sal islands (Tennent &

Russell 2015, 2019). There are other Cabo Verdean records from the islands of Santiago (Báez & García 2005) and Santo Antão (Nyström 1958; Vieira 2008), however, Tennent & Russell (2015, 2019) consider that these could be misidentifications requiring confirmation based on the more humid habitats that exist on those areas. The tenebrionid was collected in Pedra Lume, Sal (Figure 1B), where this Pieridae is common (Tennent & Russell 2015, 2019) and was previously reported in December. We captured the specimen of *Oxycara* in January. The climatic conditions in Cabo Verde have remained largely unchanged on this very arid island from 2017 (year in which previous surveys were conducted) to 2023 (year of this surveys), so it is likely that this Lepidoptera is still common in the region during this season. Species of *Pontia* have a short adult life span, less than a week for some species (Kingsolver 1999; Sidhu et al. 2014). Based on that, at least part of the *P. glauconome* population would be dying by January.

Tenebrionidae are mainly detritivores, that means that they feed mainly on decaying organic matter, such as leaves, dead wood and other plant debris (Watt 1974; Matthews et al. 2010). However, diet of Pimeliinae is quite diverse and often includes animal matter, such as dead bodies of other invertebrates (Fattorini 2023). In desert environments, where food availability can be highly variable, tenebrionids consume those whenever available, showing that these species of Coleoptera can exploit a wide range of resources available if needed (Duncan et al. 2002). Since there is no available information on the diet of *Oxycara*, this result suggests that *O. richardi*, a flighless ground dweller tenebrionid that cannot predate on living Pieridae, is feeding opportunistically on dead *P. glauconome* as part of its winter diet.

Finally, regarding to the phylogenetic tree, it seems that the taxonomy of *Pontia* is not clear and specific studies would be needed to clarify the relationships between species and the diversification within the genus (Chew & Watt 2006).

**Acknowledgements**

This work was funded by Portuguese funds through 'Fundação para a Ciência e a Tecnologia, I.P' (FCT) that funded the BIGFIT project (EXPL/BIA-EVL/0470/2021) to RV, and by the Spanish Government project-grant PID2019-110243GB-100/ AEI/10.13039/501100011033 (Ministerio de Ciencia e Innovación) to MGP. FCT also supported RV contract under the 'Norma Transitória' (DL57/2016/CP1440/CP1646/CT0003), and PJA PhD grant (2022.14742.BD; financed by the European Social Fund and the national programme 'Portugal 2030'). We also thank to Direcção Geral do Ambiente, MAA, Cabo Verde Government for the sampling and exporting permits (Nº 017/DNA/2023) and Cabeólica S.A. for supporting fieldwork.

## Conflict of interest

The authors declare that there is no known financial interest or personal relationships that could have influenced the work presented in this article.

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(Recibido para publicación / *Received for publication* 14-X-2024)
(Revisado y aceptado / *Revised and accepted* 20-XI-2024)
(Publicado / *Published* 30-IX-2025)

# COMITÉ PARA LA PROTECCIÓN DE LA NATURALEZA, PROYECTO DE INVESTIGACIÓN CIENTÍFICA DE SHILAP / *COMMITTEEFORTHEPROTECTION OF NATURE, SHILAP SCIENTIFIC RESEARCH PROJECT*

**Solicitud de autorización para recoger Lepidoptera con fines científicos en España**

Las solicitudes cumplirán las siguientes condiciones:

1. – Estar al día en el pago de la cuota anual de la Sociedad,antes de solicitar los permisos.
2. – Enviar un correo electrónico al Secretario General de SHILAP con todos los datos personales, incluyendo nom-bre, apellidos, dirección, DNI o número de pasaporte, número de teléfono (con código del país y prefijo) y correo electrónico. Estos datos serán enviados al Secretario General con un mínimo de 45 días de antelación al período de captura previsto.
3. – Se detallará el área donde se desea capturar el material (provincia y/o región), el período de tiempo (días, meses o todo el año); método de captura que se desea emplear (manga entomológica, grupo electrógeno, etc.), material que se desea recoger (especies, géneros, familias, y/o superfamilias) y cualquier otro dato que se desee añadir.
4. – Todos los socios de SHILAP que soliciten estos permisos para recoger Lepidoptera en España con fines científicos, serán incluidos en el Proyecto de Investigación Científica creado por la Sociedad y denominado: *"Faúnula Lepidopterológica Ibérica, Baleárica y región Macaronésica"*.
5. – Con el fin de contribuir con este Proyecto Científico, se ruega remitan a SHILAP, **o una copia por correo electrónico (e-mail), con el listado del material recogido en EXCEL,** sólo en este formato, indicando la Familia, Subfamilia, Tribu, nombre de la especie (género, especie, autor y año), localidad, coordenadas UTM (1 X 1) o GPS, provincia, fecha de captura, colector y número de machos y hembras capturados (**sólo 5 ejemplares por taxón y localidad, máximo**). Por favor, utilice sólo el *"Catálogo sistemático y sinonímico de los Lepidoptera de la Península Ibérica, de Ceuta, de Melilla y de las islas Azores, Baleares, Canarias, Madeira y Salvajes (Insecta: Lepidoptera)*" (A. VIVES MORENO, 2014)". Esta lista es necesaria para este Proyecto Científico de SHILAP y para nuevas autorizaciones.
6. **–Es obligatorio publicar en** *SHILAP Revista de lepidopterología,* las nuevas especies o subespecies que se descubran y remitir a SHILAP **una parte del material TIPO**, para su posterior incorporación a la colección de Lepidoptera del Museo Nacional de Ciencias Naturales en Madrid, España.
7. – Se recuerda a todos los socios de la obligación de estar autorizados para recoger Lepidoptera, con fines científicos, en España y que está prohibida todo tipo de actividad comercial, con el material capturado.
8. – Conocer los fines científicos de SHILAP y comprometerse a pagar los gastos de participación en este Proyecto Científico, que la Junta Directiva considere en cada momento.

**Application for permits to collect Lepidoptera in Spain for scientific purposes**

Applications must abide by the following conditions:

1. – The Society's annual fee must be paid before applyin for the permits.
2. – To send an electronic mail the General Secretary o SHILAP, with all the personal data, including name, sur-name, address, ID card number or Passport number, telephone number (with country code and prefix) and electronic mail address. These data must reach the General Secretary at least 45 days in advance of the foreseen collecting activity.
3. – The collecting area to be visited by the applicant should also be detailed (province and/or region), expected dates (days, months, or the whole year), collecting method (entomological net, generator, etc.), taxonomical groups of interest to be collected (species, genera, families and/or superfamilies); any other data the applicant wishes to add.
4. – All members of SHILAP who apply for these permits to collect Lepidopterain Spain with scientific purposes, will be included in the Scientific Research Project created by the Society and called: *"Lepidopterological Fauna of the Iberian Peninsula, Balearic Islands and Macaronesianregion"*.
5. – In order to contribute to this Scientific Project, it is requested to send to SHILAP, **either a copy by electronic mail (e-mail), with the listing of materials collected in EXCEL (-**only in this format, please), indicating the Family, Subfamily, Tribe, name of the species (genera, species, author's name and year), town, UTM (1X1)or GPS coordinates, province, dates of capture, collector and numbers ofmales and females captured (**only 5 specimens per taxonand locality, maximum**). Please, use only the*"Catálogo sistemático y sinonímico de los Lepidoptera de la Península Ibérica, de Ceuta, de Melilla y de las islas Azores, Baleares, Canarias, Madeira y Salvajes (Insecta: Lepidoptera)* (A.VIVES MORENO, 2014)". This list is necessary for this Scientific Project of SHILAP and for new authorizations.
6. **– It's obligatory to publish in** *SHILAP Revista de lepidopterología*, the new species or subspecies that are discovered and to remit to SHILAP **a part of the TYPE material**, for later incorporation into the Lepidoptera Collectionof the National Museum Natural Sciences, Madrid, Spain.
7. – All members are kindly reminded of the obligation to be duly authorized for collecting Lepidoptera, with scientific purposes, in Spain and that it is forbidden all type of commercial activity, with the captured material.
8. – To know about the scientific aims of SHILAP and to commit to pay the expenses of participation in this Scientific Project, that the Board of Directors considers at any given moment.