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# Genetic (RAPD) diversity between *Oleria onega agarista* and *Oleria onega* ssp. (*Ithomiinae*, Nymphalidae, Lepidoptera) in north-eastern Peru

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**Abstract**

*Oleria onega agarista* Felder and Felder and *Oleria onega* ssp. nov. are two *Ithomiinae* subspecies from north-eastern Peru, that differ for some morphological and behavioural traits. Two contact zones are known near the town of Tarapoto: Ahuashiyacu, where both subspecies cohabit but do not seem to hybridise, and Estero (near the village of Shapaja), where they apparently hybridise. Genetic differences between the two subspecies and between populations were investigated with random amplified polymorphic DNA (RAPD) markers. Both Cluster and Principal Coordinates Analyses (CCoA and PCoA) performed using these data, provided a clear but weak discrimination between the two subspecies. Genetic diversity is much higher within the populations than between them. Moreover, the geographically more distant populations are grouped together by the genetic data. Morphological traits on the wing patterns of the hybrids are intermediary between the two butterflies subspecies, while RAPDs data place them closer to *O. onega agarista* than to *O. onega* ssp. The individuals of the Ahuashiyacu population are clearly separated into two groups, those of *O. onega* ssp. and *O. onega agarista*, by both morphology and RAPDs data. Moreover, none of those individuals show RAPD similarity with the hybrids, suggesting that hybridisation has not occurred in this population.

## Introduction

It is widely accepted that the distribution of species is influenced, among others, by three major processes: speciation, extinction and transformation (e.g., Gaston, 1998). As a general picture, species having a larger distribution range, also have a greater probability to be divided by a barrier (climatic, geographic, environmental or other), than species having a small range do. Hence, the former are more susceptible to speciation processes. The frontier where two partially interfertile population or species meet (e.g., two different mimicry forms of unpalatable prey taxa), is known as a 'hybrid zone'. Usually very stale, a hybrid zone is a cline or set of clines between two parapatric or sympatric hybridising taxa (Hewitt, 1988), while contact zones are sites were two species meet without hybridisation. Because of the strong frequency dependent selection on rare forms, hybrid and contact zones are generally narrow (5–10 km) (Barton & Hewitt, 1985, 1989; Mallet, 1989; Jiggins et al., 1996). Hybrid zones are maintained by two antagonistic processes: on one hand selection (Mallet & Barton, 1989), ecological factors and adaptations (Jiggins et al., 1996) which act against gene flow, and on the other hand by dispersal that tend to lead to speciation.

In this study, we investigated the taxonomical status of two *Ithomiinae* subspecies and their morphological hybrids. We concentrated on *Oleria onega* ssp. nov, currently undescribed (Lamas, personal communication), and *Oeria onega agarista* Felder and Felder. These butterflies inhabit the easternmost mountain chain of the Andes before the Amazonian plain, in north-eastern Peru, near the small town of Tarapoto. This mountain chain, called the Cerro Escalera, is considered an ecological barrier for various organisms,

principally butterflies (Mallet, 1989, 1993; Schulte, 1999; Joron, 2000; Joron et al., 2001). On the Tarapoto side, where the climate is hotter, we found *O. onega* ssp. while only *O. onega agarista*, the subspecies of the lowland forests, was found on the other slope characterised by a wetter and cooler climate. The two subspecies seem to cohabit in only two locations: Estero, near Shapaja, and the Bocatoma of Ahuashiyacu, near Tarapoto (Figure 2). In this latter site, both subspecies are present but apparently do not hybridise, whereas in Estero morphological hybrids were observed. Therefore, Ahuashiyacu could be considered as a 'contact zone', while Shapaja could be an 'hybrid zone'.

*O. onega agarista* and *O. onega* ssp. are considered as conspecific by Lamas (Dr Gerardo Lamas Müller personal communication). However, the status of *O. onega* ssp. as a subspecies or a completely different Oleria species, is not yet established. Morphologically, *O. onega* ssp. differ from *O. onega agarista* by the narrower black edge of the wings, and by two black bands on the hindwing that are never connected (Lamas, personal communication), whereas in *O. onega agarista* a transversal band connects these two bands on their middle part on the Cu1 and Cu2 veins (Figure 1). The morphological hybrids are recognisable by an incomplete or absent transversal band. As hybrids are not frequent in the zones where both subspecies were found, we can suppose that a biological barrier exists and acts against crossings. Therefore, in a previous study (unpublished data), we put the two subspecies together to interbreed in laboratory, searching for eventual pre- or post-zygotic barriers. However, females born in cages never accepted males, regardless of whether they were from the same subspecies or not, and regardless of whether males were wild or born in cages. Behavioural differences between *O. onega* ssp. and *O. onega agarista* were also observed in the field. For example, females of the former subspecies lay eggs up to 1 m away from the host plant, whereas *O. onega agarista* lay them mostly on the leaves of the host-plant (unpublished data).

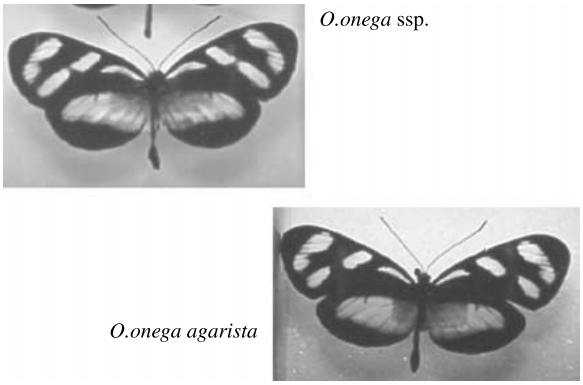


*Figure 1.* Wing patterns of *O. onega agarista* and *O.onega* ssp. showing the two black bands on the hindwing connected by a transversal band in the first subspecies and separated in the latter.

In order to better understand the genetic relationship between these two subspecies, it is important to assess the pattern of genetic variation within and between subspecies. A suitable tool is the RAPD technique, which provides a virtually unlimited number of neutral DNA markers (Williams et al., 1990) and is therefore an appropriate method for initial, overall analysis of variation between populations. In addition, material for RAPD analyses can be collected in the field and stored in alcohol, avoiding the necessity for freezing or immediate processing, required for other techniques like isozymes (Bartish, Jeppson & Nybom, 1999). Moreover, RAPD markers can easily detect differences among populations and species of different organisms, including plants (Ayres et al., 1999; Bartish et al., 1999; Bussell, 1999; Skotnicki, Ninham & Selkirk, 1999; Comes & Abbott, 2000; Guadagnuolo et al., 2001a, b), invertebrates (Moya et al., 2001; Ritchie, Kidd & Gleason, 2001) and vertebrates (Clausing, Vickers & Kadereit, 2000; Vucetich et al., 2001). Two major and often mentioned drawbacks of RAPDs markers are their lack of reproducibility and the loss of complete genotypic information, due to the fact that most RAPD bands are dominantly inherited. However, the problem of non-reproducible fragments can be highly reduced by using only high-quality DNA and by careful optimisation of the PCR conditions (Wiesing et al., 1994; Guadagnuolo et al., 2001c). Analysis of molecular variance (AMOVA), which is not influenced by the dominance of the used markers, can be used to determine the partitioning of RAPD variation between and within populations (Huff, Peakall & Smouse, 1993). Moreover, it has already been demonstrated that alternative markers, such as microsatellites, are extremely difficult to obtain for lepidopterans (Nève & Meglecz, 2000).

The aims of this study were: (i) to elucidate, at least partly, the taxonomical relationships between the two subspecies by investigating the genetic similarity between them; (ii) to investigate the molecular variation among populations and (iii) to determine the

status of the hybrids in relation to *O. onega* ssp. and *O. onega agarista*.

## Materials and methods

### *Populations sampling*

A total of 120 samples of *O. onega* ssp., *O. onega agarista* and putative hybrids between them were collected from seven populations near Tarapoto and on the Cerro Escalera (Figure 2 and Table 1). In order to evaluate genetic distances between the two subspecies, we also collected seven specimens of a different *Oleria* species, *O. gunilla serdolis* Haensch, and five specimens of a different *Ithomiinae* genus, *Hyposcada zarepha flexibilis* Haensch.

Three different conservation methods of the butterflies were tested: (i) dried and conserved in well closed boxes with silicagel, (ii) conserved in 70% alcohol, and (iii) in 99% alcohol.

### *DNA extraction*

Samples conserved in alcohol were washed three times with deionised water, and then dried with paper.

In order to obtain enough DNA for numerous RAPD reactions, the DNA extractions were performed on abdomens, which contain a large number of cells and are consequently rich in DNA. However, the ends of abdomens were removed to avoid possible contamination by the spermatophore of male origin in females, as well as the sclerified parts of male abdomens (Aubert et al., 1999). The abdomens were ground in 2 ml Eppendorf tubes containing liquid nitrogen. Extractions were then performed using the QIAGEN DNeasy Kit for animal cells (QIAGEN Inc.) according to the manufacturer's instructions and DNA was resuspended in TE (pH 8, Tris 10 mM, EDTA 1 mM). Because of degradation of the dried material, DNA extractions were finally performed only on the 133 butterflies conserved in alcohol. DNA integrity was tested on a 0.8% agarose gel, and only 96 out of the 132 extracted individuals were suitable for subsequent RAPD analyses. DNA was quantified using a WPA lightwave S2000 spectrophotometer and samples were then diluted to a concentration of 5 ng/rmul and stored at −20°C.

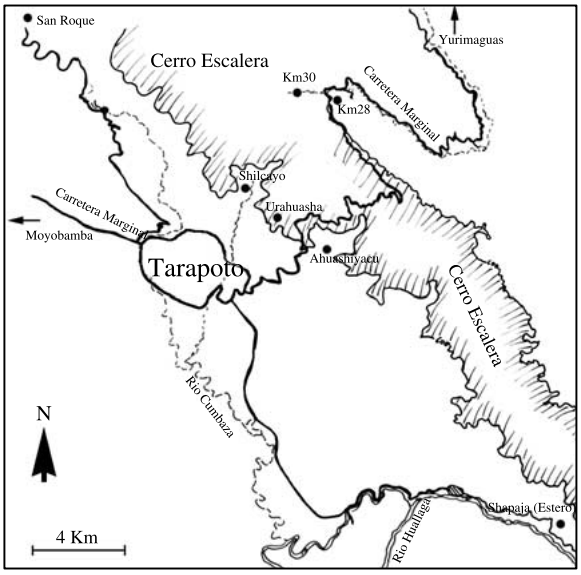


*Figure 2.* Geographical distribution of the sites sampled for *O. onega* ssp. and *O. onega agarista* in north-eastern Peru. Main roads are represented by thicker lines.

### *RAPD markers amplification*

All the PCR reactions were carried out in a final volume of 25 µl containing 25 ng genomic DNA, 2.5 µl 10× PCR buffer (with 1.5 mM $MgCl_2$), 0.25 µl 10 mM dNTPs, 0.5 µl primers (10 pmol/µl), and 0.1 µl Qiagen Taq polymerase (QIAGEN Inc). Amplifications were performed with a Biometra T-Gradient thermocycler with the following profile; 3 min at 94°C, followed by 39 cycles at 94°C for 45 s, 40°C for 45 s, 72°C for 1 min and a final extension step of 10 min at 72°C. PCR products were mixed with 1/5 volume of loading buffer and separated on a 1.2% (w/v) agarose gel, containing 0.4 µg/ml ethidium bromide, in 0.5× TBE at 100 V for 1 h. DNA fragments were then visualised under UV light.

We tested 23 random primers of the series OPB, OPP and OPT (Operon Technologies, Alameda California), on seven samples of the two subspecies using different amplification conditions, and we repeated each amplification three times to ensure the reproducibility of the results. Negative controls were added at each PCR. Seven of these primers, OPB-01, OPB-11, OPB-12, OPB-15, OPT-01, OPT-05, OPP-04, gave clear and reproducible results and were thus used on all samples, whereas OPB-04, OPT-04, OPP-06 were not taken in account because of differences between replicates. Finally OPB-02, OPB-05, OPB-06, OPB-13, OPB-14, OPB-16, OPT-02, OPT-03, OPP-01, OPP-02, OPP-03, OPP-05 and OPB-03

*Table 1.* Sampling sites of *O. onega* ssp., *O. onega agarista*, *O. gunilla serdolis*, and *H. zarepha flexibilis*, number of analysed individuals and abbreviations used in the text and figures

| Population site | *O. onega* ssp. | | *O. onega agarista* | | *O.onega* ssp./ *agarista* hybrids | | *O. gunilla serdolis* | | *H. zarepha flexibilis* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. of ind. | Abbr. | No. of ind. | Abbr. | No. of ind. | Abbr. | No. of ind. | Abbr. | No. of ind. | Abbr. |
| Shilcayo | 15 | S | – | – | – | – | – | – | – | – |
| Urahuasha | 7 | U | – | – | – | – | – | – | – | – |
| Ahuashiyacu | 11 | A | 2 | AA | – | – | – | – | – | – |
| San Roque | 13 | SR | – | – | – | – | 6 | SRogs | 2 | SHhz |
| Shapaya | 9 | SH | – | – | 7 | HSH | – | – | 1 | SRhz |
| Km30 | – | – | 18 | K | – | – | – | – | – | – |
| Km28 | – | – | 5 | KM | – | – | – | – | – | – |

were discarded because either they did not amplify any fragments, or produced only monomorphic bands.

*Data analysis*

RAPD markers were scored in a binary form as presence or absence of amplified bands (respectively 1 and 0) for each sample.

*Cluster analysis*

We performed cluster analyses using the CLUSTER package (http://www.biology.ualberta.ca/jbrzusto) to determine if samples of the same species formed groups according to their morphological appearance. Since, the shared absence of a band in two samples is rather an absence of information than an element of similarity, the asymmetrical Jaccard's coefficient, which considered only shared presence, was used to calculate similarity between samples and to generate a similarity matrix. This latter was then used to produce an UPGMA (Unweighted Pair-Group with Arithmetic averaging) dendrogram, visualised with TREEVIEW (Page, 1996)

*Principal coordinates analysis (PCoA)*

The similarity matrix calculated with Jaccard's similarity coefficient between RAPD's samples was converted into a distance matrix ($D = 1-S$). The distance matrix was used to perform PCoA using the R4 (Beta version) package (P. Casgrain and P. Legendre, Université de Montréal). Results were graphically represented in a bivariate Scattergram using StatView (SAS Institute, Inc.)

In order to test the statistical significance of groups determined by both cluster and PCoA analyses, a Mantel test was performed (999 permutations) using the R4 (Beta version) package. The similarity matrix obtained by genetic data (excluding the outgroup species *O. gunilla serdolis* and *H. zarepha flexibilis*), and a generated matrix in which a distance value of 1 was assigned between two samples of a same subspecies and a value of 0 was assigned between two samples of different subspecies, were converted into a distance matrix and compared pairwise.

*AMOVA*

AMOVA (Excoffier, Smouse & Quattro, 1992) was performed using the software ARLEQUIN 2.000

*Table 2.* Characteristics of fragments amplification by seven oligonucleotides primers in the RAPD analysis of nine *O. onega* populations

| Operon primer code | Polymorphic fragments | Mean no. of polymorphic fragments per population |
|---|---|---|
| OPB-01 | 10 | 02.89 |
| OPB-11 | 12 | 06.22 |
| OPB-12 | 12 | 03.26 |
| OPB-15 | 14 | 06.89 |
| OPT-01 | 18 | 08.55 |
| OPT-05 | 16 | 08.31 |
| OPP-04 | 6 | 01.89 |
| Mean | 11.44 | |
| Total of polymorphic fragments | 88 | |

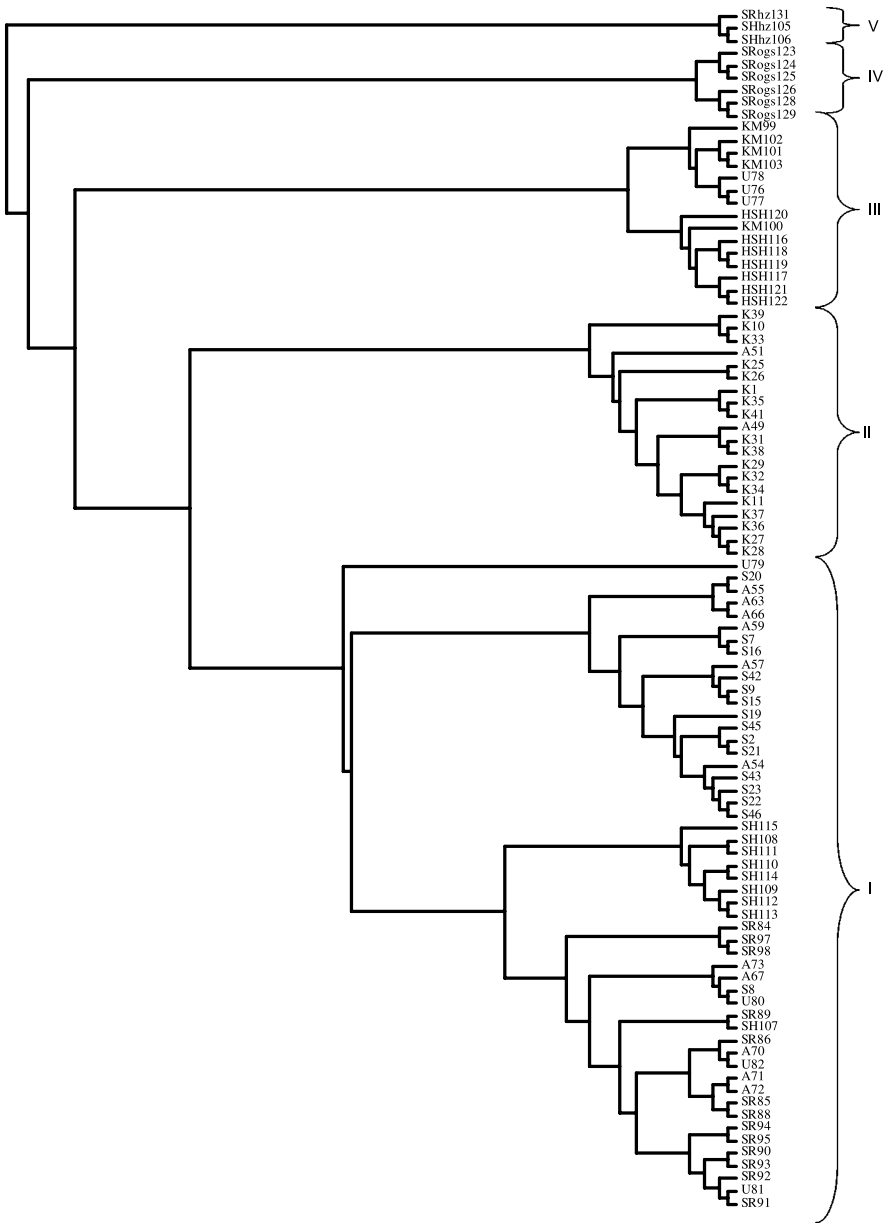


*Figure 3.* UPGMA dendrogram based on Jaccard similarity coefficient obtained for *O. onega* ssp., *O. onega agarista*, *O. gunilla serdolis*, and *H. zarepha flexibilis* using RAPD data. Group I is constituted by *O. onega* ssp., group II by *O. onega agarista*, group III by hybrids and one *O. onega agarista* population, group IV by *O. gunilla serdolis* and group V by *H. zarepha flexibilis*.

(Schneider, Roessli & Excoffier, 2000) in order to describe the genetic variability among and within subspecies and populations. Only the data for the two *O. onega* subspecies were considered in these analyses, avoiding hybrids and the outgroup. One population of *O. onega agarista*, that of Ahuashiyacu, represented by fewer than five individuals was not taken into account.

## Results

### *RAPD markers*

A total of 92 polymorphic fragments were scored, the size of which ranged between 300 and 1500 bp.

Four of the total of 92 fragments were present only in the outgroup species. Thus, out of the remaining

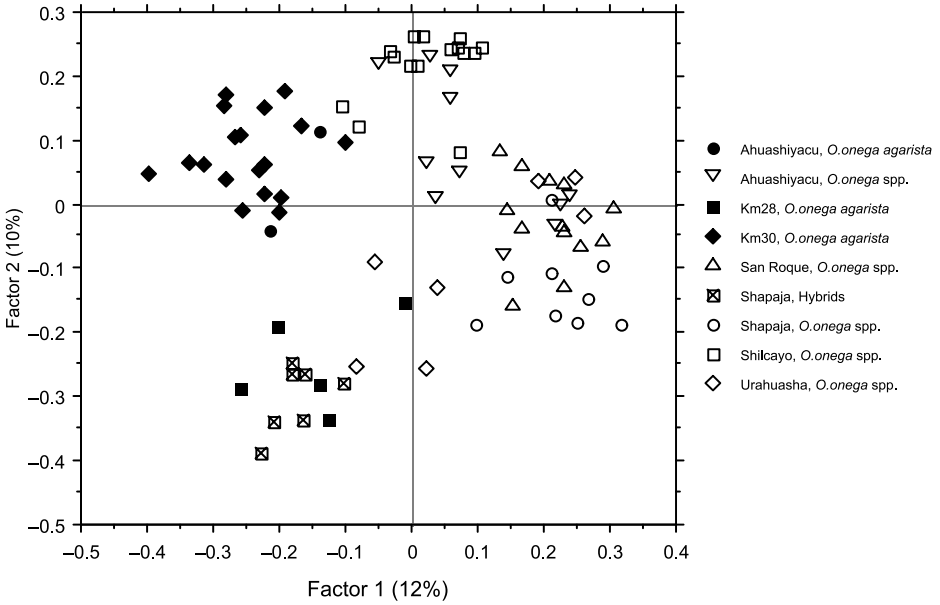


*Figure 4.* Principal coordinates analysis for *O. onega agarista* and *O. onega* ssp. based on RAPD data. The distance matrix is obtained after linear conversion of the Jaccard's coefficient based similarity matrix.

88 fragments present in the *O. onega* complex only, 76 were present *in O. onega* ssp. and 69 in *O. onega agarista*. Out of the 57 fragments common to *O. onega* ssp. and *O. onega agarista*, 30 were present in the hybrids. Five fragments specific to *O. onega* ssp. and only two specific to *O. onega agarista* were found in hybrids. In some cases, fragments that were scarce in the subspecies, were frequent in the hybrids, but no fragment was specific to hybrids.

The primers used varied widely in their ability to detect variation between and within populations. Indeed, the mean number of polymorphic fragments among populations, scored individually for each primer varied between 1.89 (OPP-04) and 8.55 (OPT-01) (Table 2).

*Data analysis*

Cluster analysis based on the RAPD-generated similarity matrix separated the individuals into five main groups (Figure 3). Group I is constituted by the different populations of *O. onega* ssp. However, while most of the individuals of the populations of San Roque (SR), Shapaja (Sh) and Shilcayo (S) are gathered in this group, those from Ahuashiyacu and most of those from Urahuasha show a higher level of within-population genetic diversity and are indeed dispersed throughout the dendrogram. Group II is composed of the *O. onega agarista* individuals from population Km30 (K) and the two individuals of *O. onega agarista* of the Ahuashiyacu mixed population (A49, A51). Hybrids from Shapaja (HSH) and the *O. onega agarista* individuals from the Km28 (KM) population, as well as some individuals of *O. onega* ssp. from Urahuasha (U76–U78) are clustered together in Group III. Finally, the groups IV and V contain the two species used as 'outgroup', *O. gunilla serdolis* (SRogs) and *H. zarepha flexibilis* (SRhz and SHhz).

The results obtained by PCoA showed that the first three principal factors accounted for 12%, 10%, and 7%, respectively, of the total variation (Figure 4). Despite these relatively low values, the two *O. onega* subspecies and their hybrids are clearly separated (Figure 4). However, as was observed with the cluster analysis, some of the individuals of each subspecies are not grouped according to their morphological appearance. Here again, hybrids are intermixed with the *O. onega agarista* individuals of population Km28, which is the geographically most distant from Shapaja.

A weak but significant correlation was observed between the genetic data and the morphological appearance of the *O. onega* individuals. Indeed, the Mantel correlation value between the RAPD's based

*Table 3.* AMOVA for RAPD phenotypes in *O. onega agarista* and *O. onega* ssp.

| Source of variation | d.f. | Variance component | Total variance (%) | *P*-value | Fixation indices |
|---|---|---|---|---|---|
| Among subspecies | 2 | 2.59 Va | 22.26 | $P < 0.001$ | $F_{ST}$0.2226 |
| Within subspecies | 84 | 9.04 Vb | 77.74 | $P < 0.001$ | |
| Among subspecies | 1 | 1.58 Va | 13.22 | $P < 0.001$ | |
| Among populations within subspecies | 2 | 2.25 Vb | 19.59 | $P < 0.001$ | $F_{SC}$0.2257<br>$F_{ST}$0.3280<br>$F_{CT}$0.1321 |
| Within populations | 71 | 7.71 Vc | 67.20 | $P < 0.001$ | |

Significance test (1023 permutations).
The total dataset contains 80 individuals from seven populations (Five *O. onega* ssp. and Two *O. onega agarista*), using 92 RAPD markers. Two analyses were conducted: the first among and within the two subspecies and their hybrids, and the second among subspecies and their populations and within populations. Percentile distribution of the variance components, as well as *P*-value and fixation indices are given. *F* statistics are defined by three fixation indices: Fct = proportion of differentiation between subspecies, Fsc = differentiation among populations within subspecies, Fst = the global differentiation of populations.

distance matrix and the generated matrix (where a distance value 1 was assigned between individuals of a different subspecies and 0 between individuals of the same subspecies) was $r = 0.14$ ($P = 0.001$).

*AMOVA analysis*

Partitioning of molecular variance was calculated among subspecies and among populations (Table 3). When using subspecies as a variable group, 22.3% of the total variability was attributable to differences among subspecies and 77.7% to differences within subspecies. Grouping the individuals according to *O. onega* geographical populations, only 13.2% of the variability was due to differences between subspecies, 19.6% to differences among populations within subspecies, and 67.20% within subspecies. The *P*-values for all analyses of variance were highly significant (Table 3).

## Discussion

*Genetic markers*

The large set of markers obtained in this study confirmed the ability of the RAPD technique to differentiate organisms at the genetic level. We detected a high level of polymorphism between the two studied subspecies, but also within each subspecies. Indeed, almost none of the amplified fragments were present in all the individuals. The high polymorphism of the studied organisms led to a difficult interpretation of the results. Indeed, only few markers were constantly present within subspecies and even populations. It was thus difficult to assess whether these fragments were specific to a subspecies or simply more frequent in some populations than in others.

Since RAPD markers are known to be highly polymorphic, it would be interesting to use more conserved markers (i.e., isozymes) or to investigate more conserved sequences (i.e., mtDNA), in order to better assess the genetic relationships between these butterflies.

*Differentiation of the subspecies and hybrids*

Both Cluster and PCoA analyses separated *O. onega* ssp. and *O. onega agarista* populations into two distinct groups. Although their classification as two different subspecies cannot be established on this basis only, the relative distance between them, lower than that separating both taxa from the different species *O. gunilla serdolis*, support this hypothesis (Figure 3).

Independent of the type of marker used, one would expect the $F_1$ hybrids between two taxa to have a position intermediate between the two studied subspecies in a genetic analysis (e.g., Guadagnuolo et al., 2001b). In the present study, the morphologically detected hybrids seem to be more closely related to *O. onega agarista* than to *O. onega* ssp. (Figure 3). The degree of hybridisation and the probability of backcrossing with either or both parents were impossible to calculate as too few hybrids were collected, and because of

the high polymorphism among individuals and populations. Nonetheless, our results suggest that at least some of the 'hybrids' could be the result a backrossing between a F1 hybrid and *O. onega agarista* as the recurrent parent.

*Hybrids occurrence and viability*

In the Ahuashiyacu population where we found both *O. onega* subspecies, no morphological hybrids were observed, whereas some were found in Shapaja. In addition, the genetic distance between the Ahuashiyacu *O. onega* ssp. and *O. onega agarista* individuals is lower than that between Shapaja *O. onega* ssp. and hybrid individuals. It is difficult to speculate on the reasons for these results, but they allow us to formulate several hypotheses. The first is that a reproductive barrier exist between the two subspecies in the population of Ahuashiyacu, that could lead to a genetic isolation. Unfortunately, it was impossible to determine whether such a barrier reducing hybridisation exists. Indeed, neither the two subspecies nor the hybrids ever reproduced in captivity, and it was thus impossible to perform crosses in laboratory. Nevertheless, natural morphological hybrids from Shapaja did not show reduced viability, and produced fertilised eggs with normal development (personal observation). These observations suggest that genetic incompatibility between *O. onega* ssp. and *O. onega agarista*, if it exists, is only partial. A second hypothesis is that barriers to gene flow are not just associated with a few strongly selected colour pattern loci, but dispersed across the genome as found on the sister subfamily *Heliconiinae* (Jiggins et al., 1997), and are a result of divergence in mate preferences, warning colour and ecology without hybrid inviability or sterility (Jiggins et al., 1996; McMillan, Jiggins & Mallet, 1997). Even so, a decisive explanation for the occurrence of hybridisation in one zone of sympatry and not in the other, is hard to give.

*Differentiation and relatedness between populations*

Because of the recent modification of the environment by extensive deforestation, with the consequent fragmentation of natural habitats, the probability of current gene flow between populations is low. The Cerro Escalera represents an additional physical barrier, which is thought to completely separate the populations of Estero from those of Tarapoto. However, our results suggest that gene exchange between populations, as well as between *O. onega* subspecies, has occurred at least in the past. The results of the AMOVA, showing a higher genetic variation within populations rather than between them, is a pattern frequently observed in both plants and animals (Skotnicki et al., 1999; Comes & Abbott, 2000; Moya et al., 2001). In the present case, this is an additional indicator of a high rate of gene flow.

The similarity of some *O. onega* ssp. individuals of Urahuasha (U76, 77, 78) with the *O. onega agarista* individuals of the Km28 population is difficult to explain, since Urahuasha is the upper population on the SW slope (Figure 2) and is separated from KM28 by the highest peaks of the Cerro Escalera. Moreover, only *O. onega* ssp. individuals were found in Urahuasha and only *O. onega agarista* at Km28. The possibility of a recent contact between these populations is thus extremely unlikely. Moreover, the other Urahuasha individuals are mixed with the Shapaja and San Roque populations, which are the geographically most distant ones. It is possible that the Urahuasha population has been in contact with *O. onega agarista* in the past. These three individuals could then be the results of hybridisation and subsequent back-crosses between $F_1$ hybrids and *O. onega* ssp. as the recurrent parent.

It should also be noted that the hybrids from Shapaja, are more closely related to the three *O. onega* ssp. individuals from Urahuasha cited above (U76–U78) than to those from Shapaja. Morphologically (data not shown), hybrids have more characters in common with *O. onega agarista* than with *O. onega* ssp. However, we found them to share five RAPD characters in common with *O. onega* ssp. versus two with *O. onega agarista*. This may suggest that the morphological characters of *O. onega agarista* are dominant over those of *O. onega* ssp., but the examination of a larger number of hybrids, as well as studies on genes coding for the morphology, are necessary to ascertain this hypothesis. On the other hand, hybridisation may lead to the introgression of only neutral alleles, leaving intact parts of the genome that are under selection and define species identity (Buerkle et al., 1999); resulting thus in organisms that show hybrid ancestry but retain the pure phenotype. Moreover, because of the selection against recombinant phenotypes, morphological measurement may consistently underestimate the true proportion of hybrids in true phenotypes (Rieseberg, Kim & Seiler, 1999a; Rieseberg,Whiton & Gardner,1999b). Another important observation is that, during the 3 years of

field work, the number of *O. onega agarista* individuals of Shapaja and Ahuashiyacu, were decreasing rapidly (data not shown). It has already been shown that extinction process may occur in five or fewer generations (Wolf, Takebayashi & Rieseberg, 2001). In order to test whether a selective pressure is acting against *O. onega agarista*, it would be interesting to genetically resample the Ahuashiyacu population, where no morphological hybrids were found, after several generations. Nevertheless, the similarity of all the *O. onega agarista* individuals of the Km28 population with the morphological hybrids could be an additional indication that support this hypothesis, since hybrid phenotype in Km28 may have disappeared even though gene information is still present. As discussed above, this population could be the result of past hybridisation events, and where selective pressure has acted in favour of the *O. onega agarista* form.

## Conclusion

The most pertinent finding of this study is the clear separation of *O. onega* in two groups. Although the high level of polymorphism within subspecies and populations make difficult the identification of individuals with a hybrid ancestry, a barrier to hybridisation, at least partial seems to exist. RAPD markers were helpful to detect genetic variation between subspecies, to identify hybrids and to describe variation within and among populations. However, in order to confirm the taxonomic statuts of each subspecies, and to measure the extent of hybridisation between them, further studies with less polymorphic markers (e.g., isozymes) or markers that allow an easier paternity analysis (e.g., microsatellites) are required. Of particular interest would also be to study of the loci coding for the colour-pattern differences and to determine whether one of the patterns is dominant over the other.

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