CROSSING EXPERIMENTS WITH SOUTH AMERICAN POPULATIONS OF NACOBBUS ABERRANS (THORNE, 1935) THORNE AND ALLEN, 1944 (NEMATODA: PRATYLENCHIDAE)

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ABSTRACT

Anthoine, G., and D. Mugniéry. 2006. Crossing experiments with South American populations of Na-


Because differing opinions have been expressed concerning the taxonomic status of Nacobbus ab-

errans, experiments based on crossing combinations of different N. aberrans populations were under-

taken. Five (N1 to N5) South American populations differing in their host preference (N1, N2, N3

able to infect potato and sugar beet; N4, N5 able to infect sugar beet, but not potato) were reciprocally

crossed. Three populations assigned to different race groups, N1 (Bolivia, potato group), N2 (Peru,

potato group) and N4 (Peru, sugar beet group) were readily able to cross between each other and to

give fertile and viable progeny, regardless of their geographical origin, race group and for most of

the crossing combination (♀/♂ × ♂/♀). When crossing a sugar beet population (unable to develop on po-

tato) as female with a male from a potato population, the progeny was able to develop on potato. The

N5 population, which belongs to the sugarbeet group from Argentina, was able to cross with other

populations, but the progenies obtained were always infertile and nonviable. This population should

be considered as a separate species. These findings provide evidence that the N. aberrans complex in

South America comprises at least two species.

Key words: amphimixis, false root-knot nematode, host preference, inheritance, molecular character-

ization, pathogenicity, races, species.

RESUMEN

Anthoine, G., and D. Mugniéry. 2006. Experimentos de cruzamiento con poblaciones suramericanas


36:67-77.

Debido a diferencias de opinión acerca del estatus taxonómico de Nacobbus aberrans, realizamos

experimentos basados en combinaciones de cruzamiento de diferentes poblaciones de esta especie.

Se cruzaron reciprocamente cinco poblaciones suramericanas (N1 a N5) con diferencias en su pre-

ferencia de hospedante (N1, N2, N3 capaces de infectar papa y remolacha azucarera; N4, N5 capa-

ces de infectar remolacha azucarera, pero no papa). Tres poblaciones asignadas a diferentes razas,

N1 (Bolivia, grupo de papa), N2 (Perú, grupo de papa) y N4 (Perú, grupo de remolacha azucarera)

d se cruzaron con facilidad y produjeron progenie fértil y viable, sin importar su origen geográfico,

raza y por la mayoría de las combinaciones de cruzamiento (♀/♂ × ♂/♀). Al cruzar hembras de una po-

blación de remolacha azucarera (inacapaz de desarrollarse en papa) con machos de una población de

papa, la progenie tenía la capacidad de desarrollarse en papa. La población N5, perteneciente al

grupo de remolacha azucarera de Argentina, se cruzó con las otras poblaciones pero las progenies

siempre fueron infértiles o no viables. Esta población debe considerarse como una especie aparte.

Estos resultados prueban que el complejo N. aberrans en Sur América está compuesto de por lo menos

dos especies.
INTRODUCTION

The nematode *Nacobbus aberrans*, originating from the American continent, is a major threat for many crops, especially for potato in Andean regions where it is well established (Manzanilla-López et al., 2002). The variability of this species has been investigated, especially in terms of morphology (Sher, 1970; Johnson, 1971; Quimi, 1981; Doucet, 1989; Doucet and Di Rienzo, 1991; Manzanilla-López et al., 1999), host range (Costilla, 1996; Jatala and Boluarte, 1993; Toledo et al., 1993), and biochemical (Doucet and Gardenal, 1992; Doucet et al., 2002) and molecular patterns (Ibrahim et al., 1997; Reid et al., 2003; Anthoine and Mugniéry, 2005a). Because of the large morphological variability among the populations of this pest occurring in North and South America, several species like *N. aberrans* (Thorne and Allen, 1944), *N. batatiformis* (Thorne and Schuster, 1956), *N. serendipiticus* (Franklin, 1959) and *N. serendipiticus bolivianus* (Lordello et al., 1961) were initially described from different geographical areas. Later, all these species were assessed as synonymous based on their morphology and defined as a single species *Nacobbus aberrans* by Sher (1970).

Jatala and Golden (1977) defined *N. aberrans* as a complex of species with several biotypes or physiological races. Recently, Reid et al. (2003) presented a new conception of the genus as a nascent complex of species based on several species *N. dorsalis*, *N. aberrans*, *N. bolivianus* and two species *inquirendae* (*N. batatiformis* and *N. serendipiticus*). A putative new *Nacobbus* taxon from Urquiza, Argentina is under formal description. The definition of *N. aberrans* is still under debate. Faced with the variability of the *N. aberrans* populations, knowledge of the existence of genetic barriers in a group of populations covering the molecular variability described for *N. aberrans* could be a useful and simple approach for the separation of potential species present in these populations. The cross-hybridization approach has been used for different complexes of species, such as *Globodera* spp. (Mugniéry et al. 1992; Thiéry et al., 1997), *Heterodera* spp. (Fox, 1967; Yeates, 1970), *Meloidogyne* spp. (van der Beek and Karsen, 1997), *Pratylenchus* spp. (Perry et al., 1980) and *Bursaphelenchus* spp. (De Guiran and Bruguier, 1989; Schauer-Blume, 1992).

According to Anthoine and Mugniéry (2005b), *N. aberrans* is an amphimictic nematode. To study the species composition of a *N. aberrans* complex, the reproductive isolation of some South American populations originating from Bolivia, Peru and Argentina was tested by crossings experiments conducted *in vitro*. The inheritance of parasitism on potato was also determined by crossing *N. aberrans* populations unable to parasitize potato with those infecting this crop.

MATERIAL AND METHODS

The *N. aberrans* populations originating from South America and isolated from infected roots (Table 1) were maintained on tomato (*Lycopersicon esculentum* Mill.) cv. Saint-Pierre under quarantine glasshouse conditions. All populations were identified as putative *N. aberrans* on the basis of their morphology, according to the description by Sher (1970) and by molecular identifi-
Crossing experiments with *Nacobbus*: Anthoine & Mugniéry

The ‘race’ group of each population was assigned, according to Manzanilla-López et al. (2002), by its parasitic ability to develop on bean (var. Triomphe de Farcy), chili pepper (var. Gros carré doux), potato (var. Désirée), sugar beet (var. Roberta), and tomato (var. Saint-Pierre). This ability was tested by inoculating the different *N. aberrans* populations composed of mixed stages (98% eggs, 2% juveniles and adults) on the differential hosts under glasshouse conditions (20°C day, 15°C night), and determining the presence of root galls containing the different developmental stages, after three months (Table 2). Extraction of stages from roots was done by grinding and centrifuging with magnesium chloride (Hendrickx *et al*., 1976). The *N. aberrans* populations used for crossing experiments were chosen according to their molecular variability, geographical origin, and race group.

**Immature Female and Male Collection and Crossing Experiments**

Reciprocal crossing experiments were conducted according to Mugniéry *et al* (1992) with adaptations for *N. aberrans*. Immature and vermiform females and males of the five *N. aberrans* populations (Tables 1 and 2) were obtained by dissecting tomato (cv. Saint Pierre) roots grown in water agar (2%) in Petri dishes and inoculated with 2nd stage juveniles (detailed

<table>
<thead>
<tr>
<th>Code</th>
<th>Location</th>
<th>Original host plant</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Bolivia</td>
<td>Potato</td>
<td>D. Cruz</td>
</tr>
<tr>
<td></td>
<td>La Paz-Aroma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>Peru</td>
<td>Potato</td>
<td>D. Mugniéry</td>
</tr>
<tr>
<td>N3</td>
<td>Argentina</td>
<td>Potato</td>
<td>M. Doucet</td>
</tr>
<tr>
<td></td>
<td>Las Estancia, Catamarca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td>Peru</td>
<td>Tomato</td>
<td>D. Mugniéry</td>
</tr>
<tr>
<td>N5</td>
<td>Argentina</td>
<td>Tomato</td>
<td>G. Karssen</td>
</tr>
<tr>
<td></td>
<td>Tucuman, Catamarca</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Code</th>
<th>Bean</th>
<th>Beet</th>
<th>Chili</th>
<th>Potato</th>
<th>Tomato</th>
<th>Race group</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>Potato group</td>
</tr>
<tr>
<td>N2</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>Potato group</td>
</tr>
<tr>
<td>N3</td>
<td>n.e.</td>
<td>n.e.</td>
<td>n.e.</td>
<td>+</td>
<td>+</td>
<td>Potato group</td>
</tr>
<tr>
<td>N4</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>Sugar beet group</td>
</tr>
<tr>
<td>N5</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>Sugar beet group</td>
</tr>
</tbody>
</table>

*Race scheme according to Manzanilla-López *et al*. (2002).*
procedure, Anthoine and Mugniéry, 2005b). Each of these vermiform females was then individually inoculated on roots of tomato cv. Saint Pierre, grown in Petri dishes. These vermiform females had not been fertilized because mating occurs after root penetration when the female becomes sedentary (Anthoine and Mugniéry, 2005b). The development of the females after their penetration in the roots was monitored until the formation of root galls and the production of the gelatinous matrices. As soon as galls and gelatinous matrix were formed, several males of the same population were placed in the Petri dish near the gelatinous matrix produced by the female until the end of the experiment. The success of the crossing attempt was assessed by monitoring the galled root inhabited by the mated female for the presence of eggs and juveniles. The hybrid status of the progeny was checked by molecular characterization (ITS pattern). When progeny was obtained, thirty specimens were selected for ITS characterization. Additional specimens were transferred to tomato roots in Petri dishes to determine their infectivity, development, and reproduction in vitro (as described above). If development and reproduction were observed, the remaining specimens were inoculated on tomato and potato for an evaluation of their parasitic capability on these crops grown in pots under greenhouse conditions.

Molecular Characterization

DNA from individuals of the different N. aberrans populations and hybrid progenies was prepared by a simplified procedure. When available, 30 specimens consisting of eggs or juveniles, of each crossing combination were individually tested. Each individual was handpicked and placed in a drop of sterile water on a glass slide. As the drop dried, the nematode was crushed between the glass slide and the cover glass by gentle pressure.

The extract was recovered with 10 µl of lysis buffer (Ibrahim et al., 1994) (10 mM Tris pH = 8.8, 1 mM EDTA, 1% Nonidet (Roche) P40, 100 µg/ml proteinase K) and incubated at 60°C for 1 h, and at 95°C for 10 min. This extract was directly used for PCR or frozen until needed.

The ITS pattern of each DNA extract was characterized using the primer set (18S-26S) developed by Vrain et al. (1992). The amplification was performed as described by Anthoine and Mugniéry (2005a). Amplification products were separated by electrophoresis on 2.5% agarose gels consisting of half Nusieve agarose (Tebu, France), and half molecular grade agarose (Qbiogène, France).

Parasitism and Host Preference Evaluation

Crossing experiments involved N. aberrans populations belonging to the potato and sugar beet ‘race’ groups, which differ by their ability to develop on potato. The sugar beet group is not able to parasitize potato. Both groups share tomato and sugarbeet as common hosts. The hybrid progeny of successful crossings was evaluated once for its ability to develop (production of egg masses) first on a common host, tomato, and then on potato as a specific host. As many juveniles as possible (all available stages) and egg masses produced as a result of several crossing combinations were inoculated on tomato and potato grown in pots under glasshouse conditions (20°C day, 15°C night). The inoculum was not calibrated, as pathogenicity was not studied. Three months after inoculation, nematode development was evaluated by gall formation and by extraction and counting of nematode life stages, including egg masses, from the roots. The paren-
Crossing experiments with \textit{Nacobbus}: Anthoine & Mugniéry

Tal populations were evaluated in the same way. The extracted hybrid nematodes were individually analyzed by ITS amplification, as previously described, to assess their hybrid status.

RESULTS

Crossing Experiments

The number of repetitions for each crossing combination was limited because of the difficulty in obtaining the right stages (females or males) at the right time. This explains why the N3 population was only used as male with the N5 population.

Reciprocal crossing experiments were performed among potato group N1, N2, and sugarbeet group, N4 and N5 populations (Table 3). Most crossing combinations among N1, N2, N4 (as female or male) succeeded in giving rise to progeny. Progenies of these reciprocal crossings among N1, N2 and N4 succeeded in building up two to three consecutive generations (Table 4).

On the other hand, when N5 was used in the reciprocal crossing experiments with N1, N2, N3 or N4 populations, the crossing was successful, especially when the N5 population was used as male. But in all crossing combinations with N5 population, the progenies were always nonviable and infertile. Few eggs were usually observed in the gelatinous matrix, except when the N4 population was used. However, no consecutive generation was obtained with these $\varnothing$ N4 $\times$ $\delta$ N5 hybrids (Table 4).

Molecular Characterization

The size of the ITS amplified fragment for all the \textit{N. aberrans} populations studied varied with several different fragments observed ranging from 900 to 950 bp (Fig. 1) with no visible variation among test replicates. N1 and N2 populations exhibited a two-band ITS pattern, whereas N3, N4 or N5 presented only a one-band pattern, which allows the identification of these populations. Because of the absence of visible variation among test replicates, DNA of each progenitor (male and female) was not used, but instead DNA from another male or female of the same population.

The molecular characterization of the hybrid progeny was undertaken for all successful crossing combinations in Table 3. This molecular characterization is illustrated by the results of the crossing combination $\varnothing$ N4 $\times$ $\delta$ N5, with each parental ITS pattern composed of a single ampli-

Table 3. Results of experimental crossings; figures show the number of crossing experiments and number of successful crossings four weeks after inoculation of males (in parentheses).

<table>
<thead>
<tr>
<th>$\delta$ population</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varnothing$ population</td>
<td>N1</td>
<td>9 (7)</td>
<td></td>
<td>4 (2*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td></td>
<td></td>
<td>5 (3)</td>
<td>2 (1*)</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>14 (7)</td>
<td>1 (0)</td>
<td></td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>4 (1*)</td>
<td>5 (1*)</td>
<td>2 (2*)</td>
<td>1 (0)</td>
</tr>
</tbody>
</table>

*Indicates progeny composed of several eggs in the gelatinous matrix, no juveniles. Where data is absent, no crossing experiments were performed.
Fied fragment of different size (Fig. 2): hybrid specimens presented a hybrid ITS pattern composed of both parental ITS amplification products. A homogeneous ITS pattern with two bands was obtained for all hybrid individuals of all crossing combinations (data not shown).

**Parasitism and Host Preference Evaluation**

The results of the parasitism evaluation (Table 5) underline that, whatever the crossing combination made among N1, N2 (able to infect potato) and N4 (unable to infect potato) populations, the progenies were viable and fertile, confirmed by the presence of eggs (Table 6) and ability to develop on tomato and potato. The ability of the hybrids to develop on potato (Tables 5 and 6) regardless of the crossing combination, suggested that this parasitic feature is dominantly inherited regardless of female or male status. The molecular characterization confirms the hybrid status of these progenies which inherited the ability to parasitize potato. It is illustrated when crossings were made between a ‘sugar beet race’ population (N4), unable to develop on potato, and a potato group population (N1), able to develop on potato and on tomato (Fig. 3). The individuals developed on potato after consecutive generations present two ITS patterns: half of the thirty individuals tested presented a two-band ITS pattern and half a single lower band ITS pattern (Fig. 3). This one-band ITS pattern may correspond to the female ITS pattern, whereas the two-band ITS pattern could either belong to a hybrid or the male pattern (the lower band of the male ITS pattern seems somewhat lower compared to all other ITS pattern of this gel). The hybrid individuals developing on potato belong to a group of individuals of different ITS pattern, not belonging to a single parental population, resulting from consecutive generations.

**DISCUSSION**

The main objective of this study was the separation of species in the *N. aberrans* complex. The crossing experiments were
undertaken with South American populations, in controlled conditions. These conditions ensured the virgin status of the females obtained and the success of crossings, as these populations were reported as obligatory amphimictic (Anthoine and Mugniéry, 2005b).

In this study, the South American \textit{N. aberrans} populations tested, except N5, interbreed readily regardless of their race group or their geographical origin. According to Mayr (1974) and his species definition, these populations do belong to a single species. On the contrary, the N5 population was not able to cross with the other populations, or if it succeeded, the progeny was not viable and fertile. According to Mayr (1974), this result sustained the hypothesis of N5 as a separate taxon, isolated from the other populations.

When considering all the successful crossing combinations among the N1, N2 and N4 populations, the molecular characterization proves the hybrid status of the progenies obtained. Nevertheless, some ITS hybrid patterns may be questionable to assess the hybrid status because of the limits of electrophoresis and the existence of two band ITS patterns. For example, when crossing a female population with a two-band ITS pattern, with a male population with a one band ITS pattern (e.g., $\varnothing$ N1 $\times$ $\delta$ N4), the ITS hybrid pattern corresponds to the female pattern. However, the presence of a viable and fertile progeny proves the success of crossing experiments, as amphimixis was previously noticed as the sole reproduction mode for \textit{N. aberrans in vitro} for these populations (Anthoine and Mugniéry, 2005b). Thus, the hybrid

![Fig. 2. Hybrid ITS pattern for the crossing combination $[\varnothing N4 \times \delta N5]$: parent (F: female, Ma: male, H1 to H10, ten different individuals of hybrid origin).](image)

### Table 5. Parasitism evaluation: ability to develop on tomato or potato.

<table>
<thead>
<tr>
<th>Host</th>
<th>Tomato</th>
<th>Potato</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental populations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>+(^1)</td>
<td>+</td>
</tr>
<tr>
<td>N2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N4</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Hybrid progenies from crossings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\varnothing N4 \times \delta N1$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$\varnothing N1 \times \delta N4$</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$\varnothing N2 \times \delta N4$</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^1\)Presence (+) or absence (—) of galls, egg masses and juveniles stages.
specimens inherit their DNA from both parents based on ITS amplification patterns, even if male’s behavior and mating were not studied. To thoroughly investigate the ITS sequences rearrangements in the hybrids, sequencing and sequence analysis would be useful and is presently under investigation.

As reciprocal crossings among the N1, N2 and N4 populations gave viable and fertile progenies, no genetic barrier seems to interfere. Although these populations are originally geographically distant and present different host range and molecular ITS patterns, they are able to cross and should be considered as belonging to a single species or a continuum of species still able to cross.

The development (the features of the life cycle, total duration) of the progenies was not investigated herein, even though some biological aspects were determined such as their parasitism and host preference. This study did not show if these hybridized progenies develop at a slower rate than that of the parental progenies. Low reproduction rate may indicate a long-term reduced viability for hybrids especially in natural conditions. In this case, these hybridized populations are produced by separated species still able to interbreed. Schauer-Blume (1992) showed successful interbreeding between *B. mucronatus* from France and *B. xylophilus* from Japan, but with less fertile progenies than for intraspecific crossings.

Table 6. Parasitism evaluation: ability to develop on tomato or potato and counting of developmental stages per plant (number of individuals).

<table>
<thead>
<tr>
<th></th>
<th>Tomato</th>
<th>Potato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eggs</td>
<td>J2</td>
</tr>
<tr>
<td>Parental populations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>16861</td>
<td>1652</td>
</tr>
<tr>
<td>N2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>N4</td>
<td>23344</td>
<td>1371</td>
</tr>
<tr>
<td>Hybrid progenies from crossings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀ N4 × ♂ N1</td>
<td>55700</td>
<td>800</td>
</tr>
<tr>
<td>♀ N1 × ♂ N4</td>
<td>15000</td>
<td>600</td>
</tr>
<tr>
<td>♀ N2 × ♂ N4</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data not available.*
Concerning the N5 *N. aberrans* population, crossing experiments succeeded in giving rise to a progeny but neither viable nor fertile whatever the crossing combination used. Karyological incompatibility as presented by van der Beek and Karssen (1997) can be rejected, as all N1 to N4 populations tested present an intra-population karyological variation (Anthoine and Mugniéry, 2005b), and are still able to cross. Thus, the crossing results suggest the existence of a genetic barrier between this N5 population and the others tested, supporting the hypothesis of a separate species. Previous molecular and karyological results have already supported N5, an Argentinean population, as a separate group (Anthoine and Mugniéry, 2005a, b). Moreover Reid et al. (2003) presented some Ecuadorian, Mexican and one Argentinean *N. aberrans* population as a separate group representing a separate species. Ibrahim et al. (1997) also underlined two distinct groups (Peruvian versus Mexican and Argentinean) based on different approaches. The ability of the *N. aberrans* populations used in the present study to interbreed with those Mexican and Ecuadorian *N. aberrans* populations remains to be investigated. The results of these experiments may help to clarify the N5 species status.

Concerning the parasitism of hybrid progenies among N1, N2, and N4 population crossings, the ability to develop on both tomato and potato was noticed for all crossing combinations. This result suggests that this ability to develop on potato is inherited as a dominant feature. The molecular characterization with the presence of different ITS patterns confirm the hybrid status of the individuals developed on potato. They do not belong to a homogenous ITS population selected from all hybrids and that developed on potato. These parasitism results underline that the hybrids immediately acquired the ability to develop on potato. However, to precisely define the risk of hybrid populations on various hosts, it would be necessary to determine their complete host range and to compare their development features with the ones of the parental populations. Irrespective of these forthcoming studies, the present parasitism results strengthen the need of strict quarantine regulations for *N. aberrans*, already implemented in many countries and especially in those, such as the United States, when the *N. aberrans* populations able to infect potato are not present (Inserra et al., 2005).

The results of crossing experiments underline that, in a set of five *N. aberrans* populations, three of these populations are able to interbreed without any genetic barrier. One Argentinean population, N3, was not studied enough to assess the existence of genetic barriers. However one population of the studied set, the N5 population is not able to giving rise to viable and fertile hybrids with these populations and thus is genetically isolated. These results support the hypothesis of at least two genetically separated groups in the *N. aberrans* complex in South America that could be recognized as two different species. The hybrid ITS patterns observed also confirmed that these *N. aberrans* populations do not reproduce parthenogenetically or by pseudogamy and corroborated the obligatory amphimixis of the false root-knot nematode as reported by Anthoine and Mugniéry (2005b).

**ACKNOWLEDGMENTS**

The authors wish to thank Dr. D. Cruz (University of San Andres, Bolivia), Dr. M. Doucet (University of Cordoba, Argentina), Dr. G. Karssen (Plant Protection Service, The Netherlands), for providing nematode populations, Drs. O. Plantard and E. Grenier (INRA, France) for their helpful reviews and suggestions.
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Crossing experiments with *Nacobbus*: Anthoine & Mugniéry


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Received: 13/IX/2005

Accepted for publication: 8/XI/2005

Recibido: 13/IX/2005

Aceptado para publicación: 8/XI/2005